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REGULAR DEPARTMENTS

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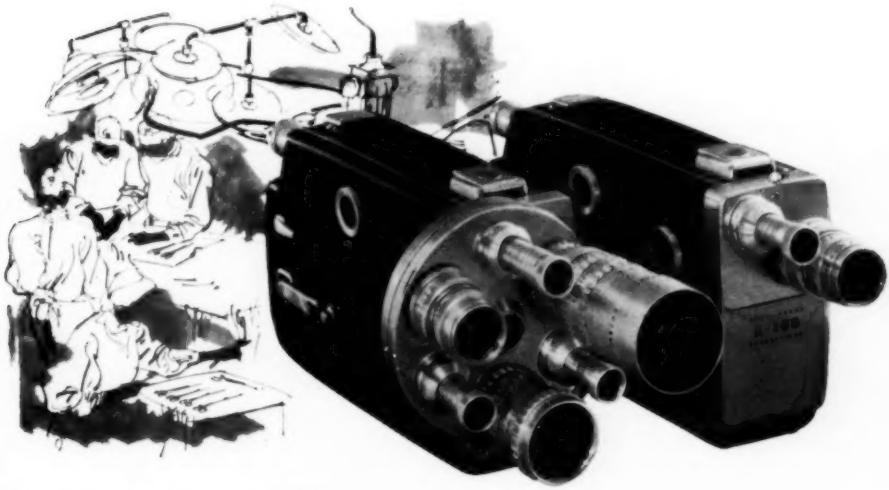

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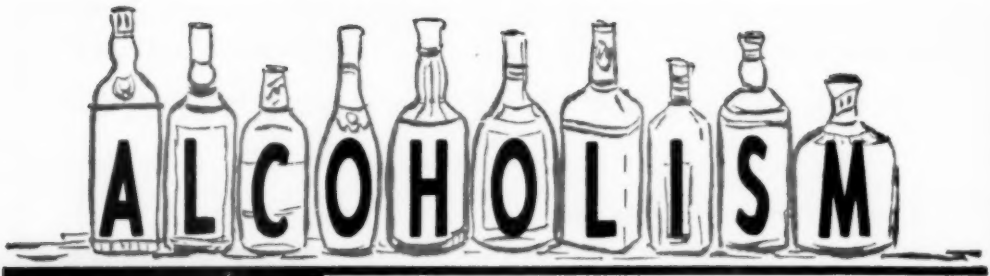

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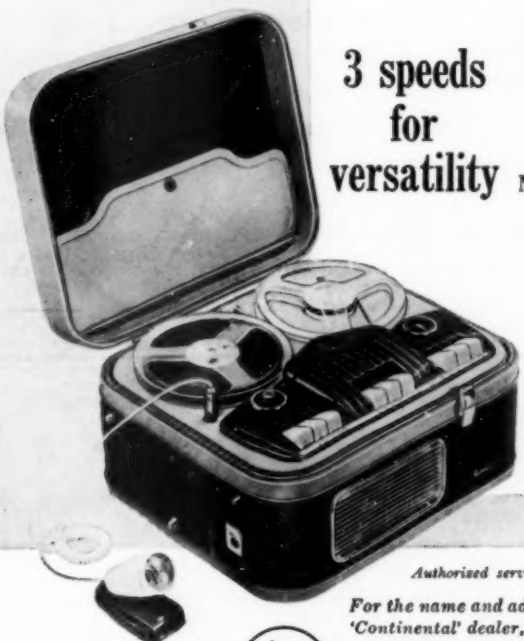

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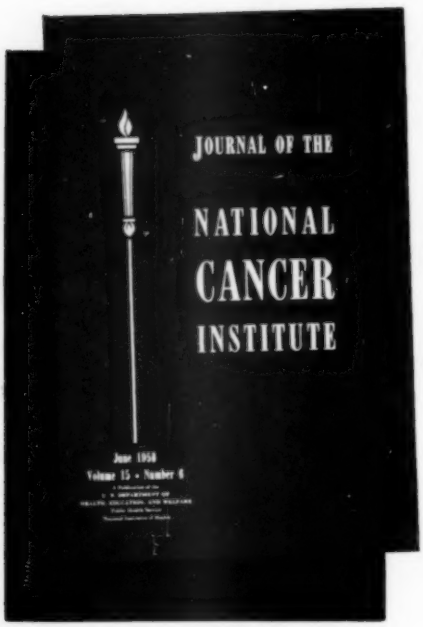

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A.M.A. ARCHIVES OF
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Bronchiolitis Obliterans

An Experimental Study of the Pathogenesis and the Use of Cortisone in Modification of the Lesions

THOMAS J. MORAN, M.D., and H. RICHARD HELLSTROM, M.D., Pittsburgh

Bronchiolitis obliterans has been described in a variety of human and experimental conditions. It has occurred after war-gas poisoning,[1] exposure to burning x-ray films,[2] exposure to fumes of nitric and sulfuric acid,[3] and working in silos.[4,5] Is has also been reported following respiratory infections, especially influenza,[6] measles, and whooping cough.[7]

The disease may be suspected when recovery from an acute respiratory illness is interrupted by the development of cough, dyspnea, cyanosis, weakness, and occasionally blood-tinged sputum. Dyspnea is usually progressive, and death often occurs in a few days to weeks. Roentgenograms of the chest show fine diffuse mottling suggesting miliary tuberculosis. In some instances a clear-cut history of antecedent disease is not obtained and a clinical diagnosis of miliary tuberculosis may be made.[8] Treatment, including use of antibiotics, has been ineffective. The use of cortisone has been suggested in silo-filler's disease,[4] but its value has not yet been established in either human or experimental disease.

At autopsy of human cases the lungs are finely nodular and the gross appearance suggests miliary tuberculosis. On close examination, however, a central slit or pinpoint opening representing a bronchiolar lumen is seen in the center of many of the nodules. The nodules, which are roughly round or ovoid, are not sharply circumscribed and extend into the surrounding parenchyma in irregular fashion, and both this feature and the bronchiolar lumens usually permit gross differentiation from tuberculosis. Microscopically the nodules consist of bronchioles or small bronchi distorted and thickened by fibrous tissue. The fibrous tissue narrows the lumen and usually surrounds the wall and extends irregularly into the lung parenchyma. The luminal narrowing in many instances is caused by polypoid fibrous projections, usually covered by epithelium, but in some bronchioles the lumen is narrowed to a slit or crescent-shaped opening by diffuse invasion of fibrous tissue without polypoid form or prominent epithelial regeneration. In some instances the lumen is completely obliterated. The lungs are emphysematous.

The nitrogen oxides have been identified as the chemical agents causing silo-filler's

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Department of Pathology, University of Pittsburgh School of Medicine and the Presbyterian, Woman's, and Eye and Ear Hospitals. Trainee, National Cancer Institute; present address: Veterans' Administration Hospital (Dr. Hellstrom).

disease[4] and the chemicals produced by burning x-ray films.[2] Fuming nitric acid, the fumes of which are nitrogen dioxide, is now used commonly as an oxidizer in rocket propellants, and this use constitutes a serious potential source of human bronchiolitis obliterans. Oxides of nitrogen are also released in many industrial processes, including the manufacture of sulfuric and nitric acid and the nitrates. According to Jacobs,[9] "as little as 0.01 per cent (100 parts per million) may cause illness if breathed for a short time and 0.07 per cent, or 700 parts per million, is fatal if breathed for 30 minutes or even a lesser time. The maximum concentration allowable for an exposure of several hours' duration is of the order of 10 parts per million."

Winternitz[10] produced bronchiolitis obliterans in dogs by exposing them to chlorine, phosgene, or chloropicrin. He postulated that the lesions were caused by long-continued probably secondary inflammation of the damaged bronchial wall. Durlacher and Bunting[11] reported "obliterative bronchiolitis" in dogs four to nine days after exposure to phosgene. Coman et al.[12] noted desquamation of bronchiolar epithelium one hour after exposure to phosgene gas and described regeneration and scarring in surviving animals. These authors felt that "the response of the lung to phosgene is a type reaction common to several other agents." Numerous other investigators have observed bronchiolitis obliterans in experimental animals, but we are not aware of additional contributions to its pathogenesis. In a previous study[13] on the effects of intratracheal injection of ethyl alcohol into rabbit lungs, we observed typical lesions of bronchiolitis obliterans and proposed the concept that the fully developed lesion was a direct result of the healing and scarring of the initially damaged portion of the bronchiole rather than of long-continued or secondary inflammation.

The present study was designed to find a simple chemical method of producing bronchiolitis obliterans, to determine the pathogenesis of the chemically produced disease, and to determine whether or not the experimental lesions could be prevented or modified by use of cortisone. Since bronchiolitis obliterans is known to follow exposure to nitrogen oxides, and since nitric acid is an aqueous solution of nitrogen dioxide, the feasibility of using intratracheal injections of dilute nitric acid as an etiologic agent in the production of bronchiolitis obliterans was considered. This technique, if successful, would be simpler and safer than using nitrogen dioxide and would avoid the necessity of a gas chamber. With this method portions of the lung could be left undamaged and development of the lesions in involved portions could be traced at various time intervals without heavy loss of animals at undesired times.

Since it has been shown[14] that cortisone delays healing by fibrosis in the rabbit, it was thought that cortisone might modify or prevent the experimental lesions of bronchiolitis obliterans by delaying fibroblastic activity until epithelial regeneration occurred. The experiment was planned so that study could also be made of the effects of cortisone and a combination of penicillin and streptomycin on the acute pulmonary edema and the chemical pneumonia produced by injection of nitric acid. Antibiotics were used so that the progression of the lesions could be followed without the presence of secondary bacterial infection.

Methods

Weak solutions of nitric acid were injected intratracheally, under light pentobarbital (Nembutal) sodium anesthesia into 110 healthy white rabbits ranging in weight from 2 kg. to 4 kg., with most animals in the 2.5 to 3.5 kg. range. A volume of 5 ml. was arbitrarily employed, because this amount of fluid covers a considerable portion of the bronchial surfaces without overflowing. A concentration of 1.0% or 1.2% nitric acid was used because these strengths were found to cause severe damage to the bronchial and bronchiolar epithelium without killing all of the animals.

The animals were divided into three groups. The first group, of 37 rabbits, received only the intratracheal injections of nitric acid. The second group, of 38 animals, was given 150,000 units of penicillin (penicillin G, Squibb) and 0.15 gm. of

a streptomycin preparation (Distrycin, Squibb) intramuscularly once daily starting the day before the intratracheal injection. The third group of 35 animals received intramuscular injections of 12.5 mg. of cortisone daily in saline (cortisone acetate; Merck Sharp & Dohme), in addition to the daily penicillin and streptomycin, beginning the day before the intratracheal injection. A few animals in the second group were given the penicillin and streptomycin for only three or four days; the others were continued on these drugs up to 14 days or until they died. Certain animals died while still receiving cortisone; the others were treated with cortisone and penicillin and streptomycin for 4 to 19 days and then killed at varying times.

Animals dying spontaneously were autopsied as soon as possible. Most of the surviving animals were killed by injection of air into marginal veins at one or two months; the rest, at periods ranging from one week to six months. Six blocks of lung including areas from each lobe were examined routinely. Additional blocks were examined when gross abnormalities were observed in areas aside from those taken for routine sections. The blocks were fixed in 4% solution of formaldehyde, and sections were prepared from paraffin-embedded material. Hematoxylin and eosin stains were made from all blocks, and selected sections were stained with Masson's trichrome method or Gram stains. Frozen sections stained by Sudan IV were made in selected cases. The findings were compared with those observed in the following experimental animals: (1) 9 rabbits which were given intratracheal injections of 5 ml. of sterile distilled water, (2) 6 rabbits which were given intratracheal injections of 5 ml. of sterile 0.9% solution of sodium chloride, (3) approximately 330 rabbits and guinea pigs given intratracheal injections of various materials in previous experiments,[10,16–18] and (4) numerous apparently normal rabbits which were killed by intravenous injections of air.

Results

In the accompanying Table a summary is presented of the mortality rate from acute pulmonary edema or pneumonia, and the incidence of focal lesions of bronchiolitis obliterans in the survivors is shown. The death rate in the untreated group was 23 of 37 (62%); in the penicillin and streptomycin group, 26 of 38 (68%), and in the cortisone, penicillin, and streptomycin group, 23 of 35 (66%). It is obvious, then, that neither a combination of penicillin and streptomycin nor a combination of cortisone, penicillin, and streptomycin influenced survival. This held true at all time intervals.

Results in 110 Rabbits Given Five Cubic Centimeters of 1% or 1.2% Nitric Acid Intratracheally

		Died		
	Total No.	6 Hr. or Less	12 Hr. to 10 Days	Bronch. Oblit. in Survivors
Nontreated	37	10	13	7 of 14
Pen. & strep.; no cortisone	38	10	16	6 of 12
Cortisone, pen. & strep.	35	10	13	1 of 12

Animals which died at six hours or less presented a different clinical and pathologic picture from those dying later. Animals dying within six hours never recovered from the initial damage and remained dyspneic and acutely ill, with progressive pulmonary edema and hemorrhage. Those surviving 12 hours or more improved after the initial effects and seemed to be recovering when death occurred unexpectedly during the first night or at periods up to 10 days. These animals apparently died of chemical pneumonia with anoxia, and they presented different histologic changes from animals dying in six hours or less of acute pulmonary edema and hemorrhage.

As shown in the Table, the incidence of focal bronchiolitis obliterans in the untreated and the penicillin-and-streptomycin-treated animals was exactly the same. Thirteen of twenty-six "non-cortisone" animals, then, showed focal but multiple lesions of bronchiolitis obliterans, compared to only one of twelve survivors in the cortisone-treated group. Two other animals in the cortisone-treated group, examined a month after injection, had a few polypoid projections filled with fibroblasts but no well-developed fibrous tissue, and several others showed epithelial regeneration, sometimes in papillary fashion, but no fibrosis.

The animals, for detailed description of the abnormal findings, fell into three categories, depending on the times and modes of death: (1) died spontaneously within 6 hours, (2) died spontaneously between 12 hours and 10 days, and (3) killed from 1 week to 6 months.

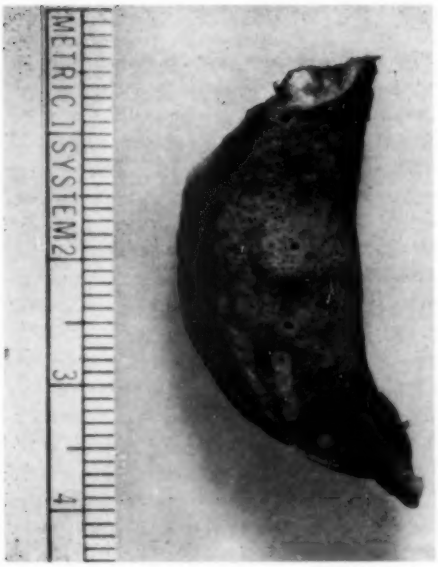

Fig. 1.—Gross appearance of rabbit lung six days after intratracheal injection of 1.0% nitric acid. Irregular nodules are seen around bronchiolar and bronchial lumens.

Spontaneous Deaths Within Six Hours.—Among 30 animals, 5 died within 3 minutes; 3, in 10 to 20 minutes; 5, in 1 to 1½ hours; 9, in 2 to 3½ hours, and 8, in 4 to 6 hours. Treatment did not influence the time of death. Death was preceded by outpouring of frothy gray, pink, or red fluid from the nose and mouth. The lungs, including trachea, weighed from 14 to 42 gm., with an average of 30 gm. (Normal weight of lungs and trachea in our animals killed by air injection is 10-14 gm.) No weight differences could be detected among the three groups, but lungs of animals dying in four to six hours were heavier on the average than those dying sooner, because of an increase in edema fluid. Gross changes in these lungs were those of congestion, edema, and hemorrhage. The trachea and bronchi were filled with frothy gray, pink, or red fluid, and the mucosal surfaces were dusky and congested. Large and small hemorrhages were scattered through the lung

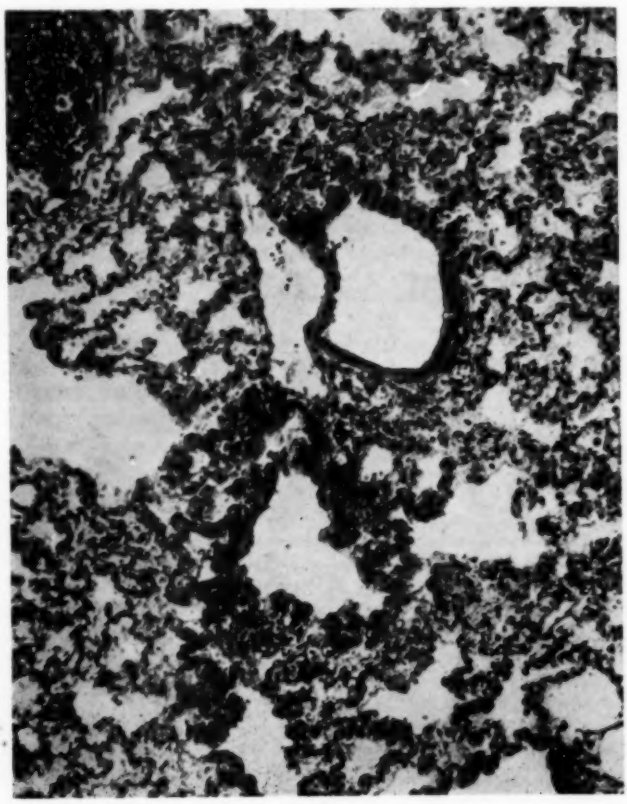

Fig. 2.—Photomicrograph of lung from a rabbit which died 10 minutes after intratracheal injection of nitric acid, showing striking localization of hemorrhage in the capillaries of the respiratory bronchioles. Hematoxylin and eosin; × 155.

parenchyma. Some of the large hemorrhagic areas resembled the "hemorrhagic infarct" appearance described by Winternitz in studies with hydrochloric acid.[19]

Congestion and hemorrhage were the only prominent histologic findings. In a number of animals dying in three minutes to one hour the hemorrhage was strikingly confined to the respiratory bronchioles, involving the capillaries just beyond the bronchiolar epithelium (Fig. 2). This was the first morphologic evidence of the extensive damage produced by the nitric acid and the initial stage in the development of the bronchiolitis obliterans.

Edema fluid was usually not prominent unless the animals lived at least two hours. Occasional macrophages and numerous red blood cells were found in the alveolar spaces, but no granulocytes were present. Bronchial and bronchiolar epithelial changes were difficult to evaluate before two hours. The first changes were in the respiratory bronchioles, but later changes appeared in different parts of the bronchial tree almost simultaneously at two to four hours. The epithelium appeared "coagulated," with the cytoplasm somewhat smudged, granular, and eosinophilic. Partial desquamation of epithelium was noted in some animals at two hours and was present in most animals at six hours. The reserve layer of epithelium was quite prominent at four hours in bronchi and bronchioles where epithelial desquamation had begun. (Fig. 3) Polypoid projections were not seen. A few veins were filled with antemortem clot in one rabbit which died in four hours.

Spontaneous Deaths Between Twelve Hours and Ten Days.—The animals dying

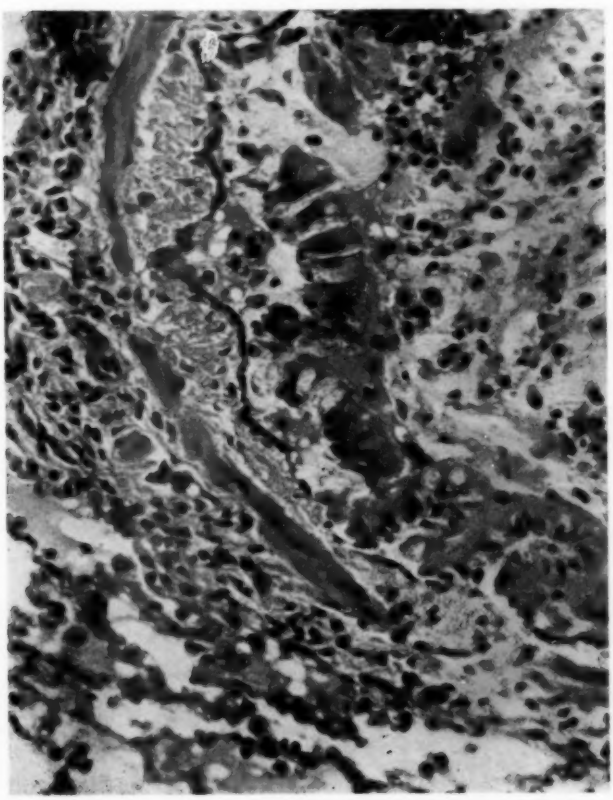

Fig. 3.—Desquamation of bronchiolar epithelium in a rabbit which died four hours after intratracheal injection of nitric acid. The dark-staining flat reserve layer attached to the wall has become prominent. Hematoxylin and eosin; × 280.

in 12 hours to 10 days showed edema, hemorrhage, pneumonia, early bronchiolitis obliterans, bronchiectasis, and abscess formation. The trachea and bronchi were congested and often contained dirty gray or yellow material. Occasional bronchi were thin, dilated, and inflamed. The earliest grossly recognizable stage of bronchiolitis obliterans was found in a rabbit which died in six days. The lungs contained numerous irregular yellow or gray nodules around bronchi and bronchioles.

Microscopic changes included edema, hemorrhage, and early pneumonia, often with hyaline membranes. At 12 hours the desquamating epithelium and lamina propria formed numerous conspicuous papillary processes in the small bronchi and bronchioles (Fig. 4). These projections were most prominent in the respiratory bronchioles, which contained a coagulum of edema fluid, fibrin, and red blood cells, often filling the projections and at times the rest of the lumen and often extending into the contiguous alveolar spaces and into larger bronchioles. The coagulum was also seen in bronchioles in which the epithelium was intact (Fig. 5). The epithelium was often arranged in irregular strands or cords which bridged the bronchial or bronchiolar lumens and produced a bizarre reticulated pattern. In some instances the entire epithelium was desquamated; at other times portions of the entire thickness of epithelium or its reserve layers were intact. The cytoplasm of much of the bronchiolar epithelium had a coagulated appearance and was deeply eosinophilic. The alveolar spaces were filled with varying amounts of edema fluid, fibrin, red blood cells, macrophages, and a few granu-

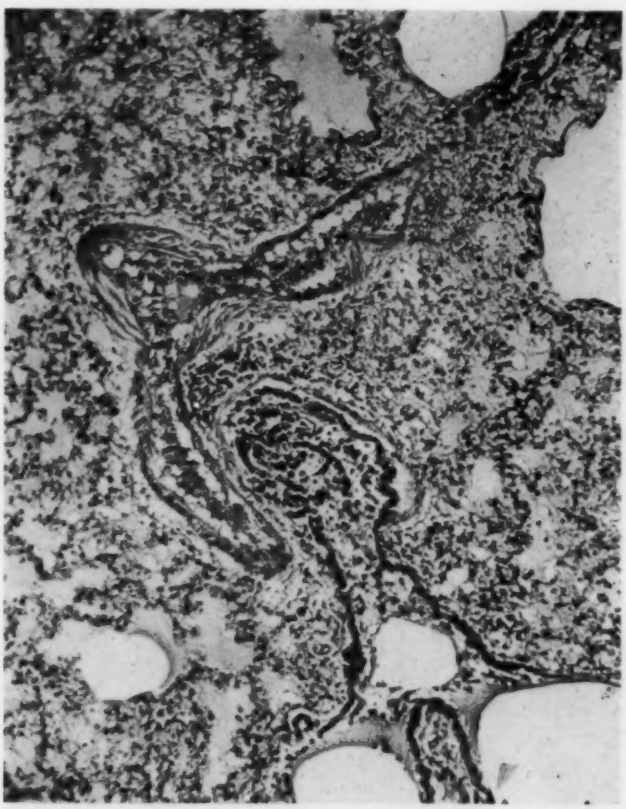

Fig. 4.—Papillary bronchiolar projections containing early coagulum in rabbit which died 12 hours after intratracheal injection. The surrounding alveoli are filled with edema fluid and a few macrophages and granulocytes. Hematoxylin and eosin; × 140.

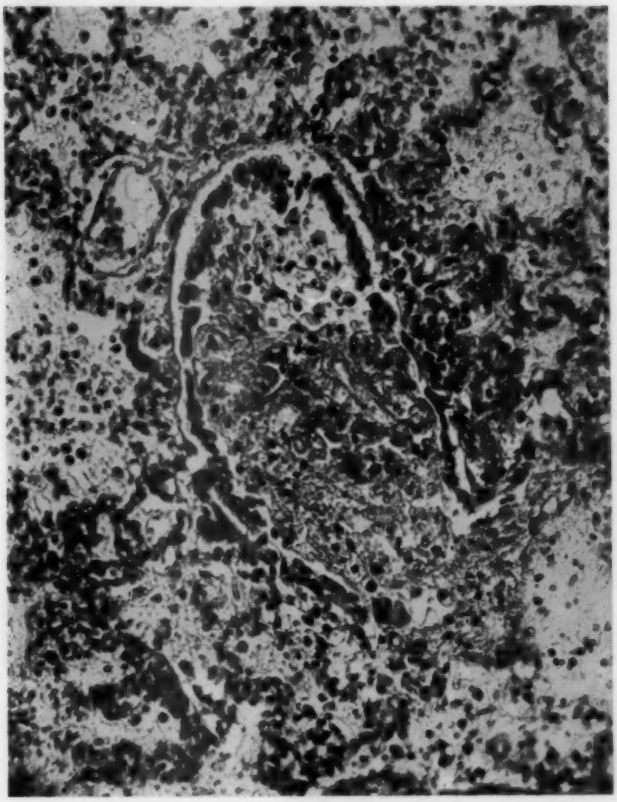

Fig. 5.—Coagulum in respiratory bronchiole and early pneumonia in a rabbit which died 24 hours after intratracheal injection. Hematoxylin and eosin; × 280.

locytes. Hyaline membranes were often conspicuous. Emphysema was present in many areas. Many bronchi and bronchioles were dilated and filled with granulocytes in 24 hours.

Partial regeneration of epithelium was present in one animal at 20 hours, and in several there was complete replacement of epithelium in some of the small bronchioles at 24 to 36 hours. The regenerating epithelium could readily be distinguished from the old epithelium by the much darker staining of its cytoplasm. The dark-staining regenerating epithelium observed in one bronchiole at 36 hours contrasted sharply with the old pink-staining desquamating epithelium that still ringed the lumen and formed a separate layer, adherent to the new (Fig. 6). All stages in the regenerating process could be observed in the same animal, and occasionally all were present in one bronchiole. The first change was a prominence of a single layer of basophilic reserve cells in which the normally flat elongated cells became plump with rounded nuclei. The cells then became cuboidal, the cytoplasm became more abundant (Fig. 7), and the cells then formed double and triple layers. Columnar cells were also seen, and by 36 hours many of these were ciliated and vacuolated (Fig. 8).

Aspirated food was found in many animals and in several probably contributed to death. A foreign-body-giant-cell reaction to the aspirated food was prominent in several animals. Arterial margination of leukocytes was well demonstrated in several rabbits. Venous thrombi were sometimes present, and in one animal both a thrombus and margination of leukocytes were found in the same artery.

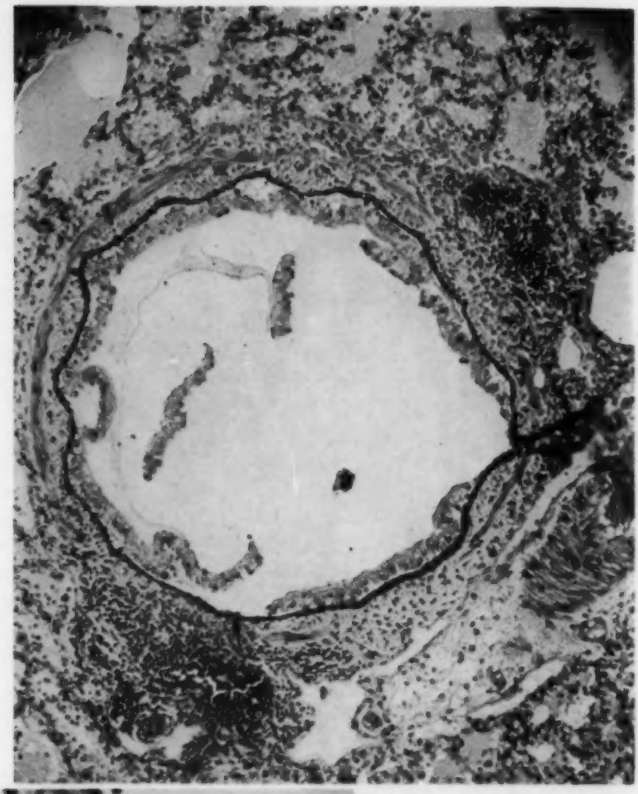

Fig. 6.—Epithelial regeneration in bronchiole of a rabbit which died 36 hours after intratracheal injection. The regenerating epithelium is seen as a dark-staining band completely encircling the wall and almost completely covered by the pale-staining desquamating epithelium. Hematoxylin and eosin; × 140.

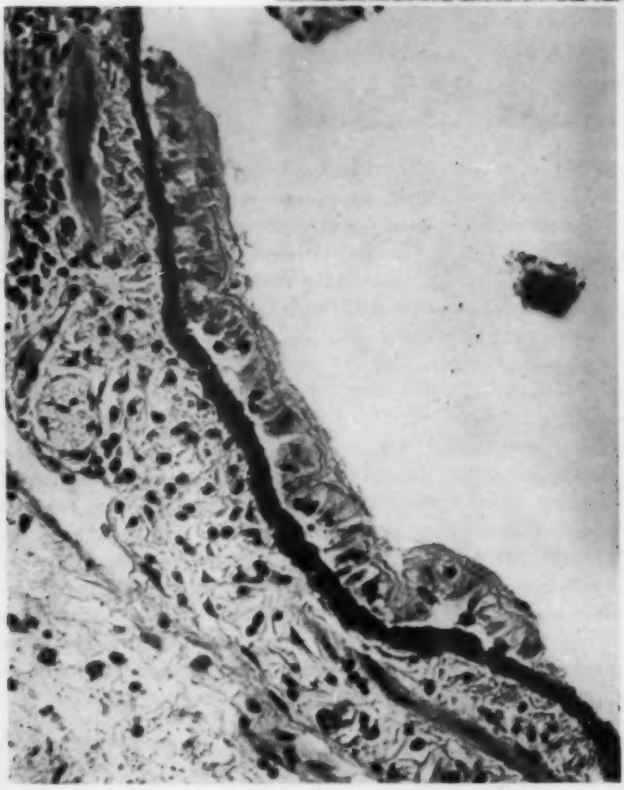

Fig. 7.—A higher power photomicrograph from an area of Figure 6, showing regenerating epithelium beneath the old pale desquamated ciliated epithelium. The regenerating epithelium in the upper portion is in a single layer, while in the midportion it is becoming stratified. Hematoxylin and eosin; × 280.

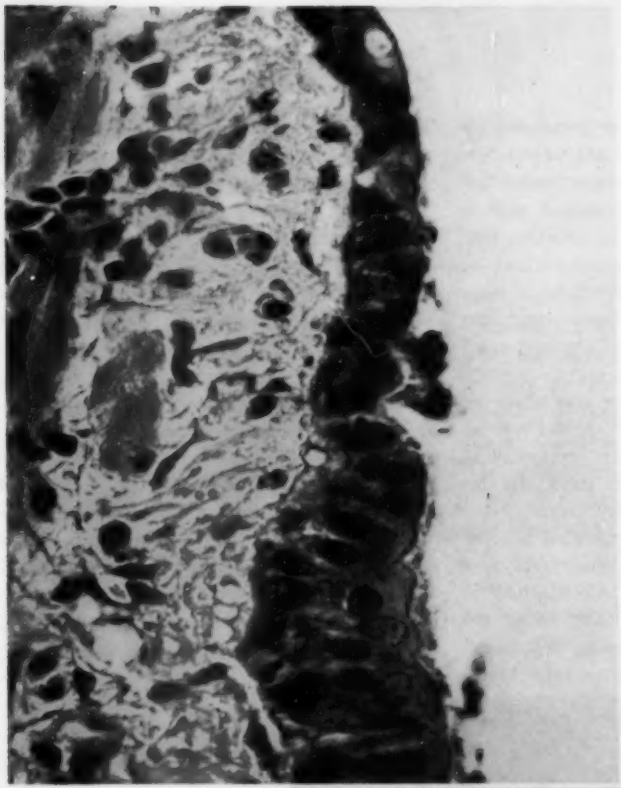

Fig. 8.—High-power photomicrograph of regenerating epithelium in same animal as Figure 6 (36 hours), with cilia and cytoplasmic vacuoles. Hematoxylin and eosin; × 800.

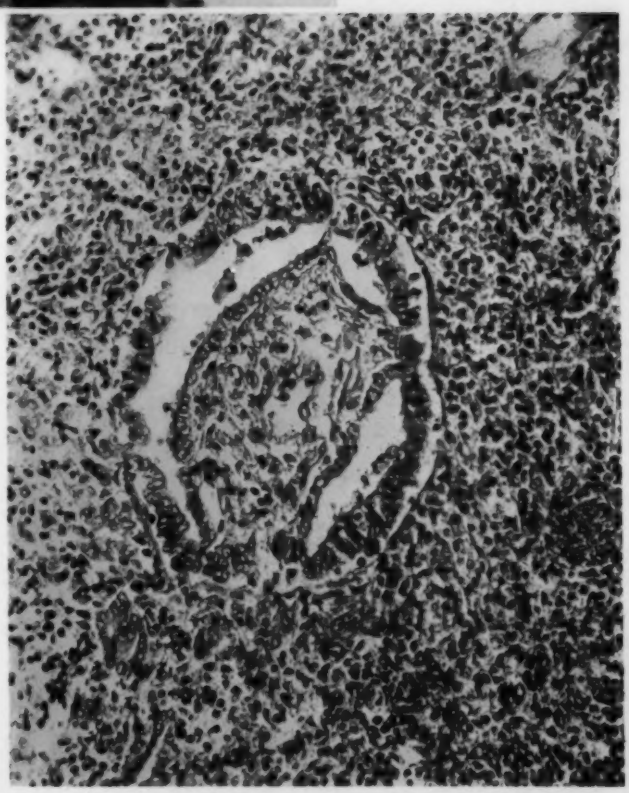

Fig. 9.—Polypoid bronchiolar projection containing fibroblasts and covered by regenerated epithelium which is attached to the lining epithelium at two other points. Rabbit died six days after intratracheal injection. Hematoxylin and eosin; × 280.

Extensive pneumonia, with occasional abcess formation, was seen in animals at 3 to 10 days. Gram stains of sections from these areas showed no bacteria, except in one instance where numerous Gram-positive rods were present. Occasional areas in each of several animals contained collections of large pale foamy cells, the appearance suggesting so-called "endogenous lipid pneumonia." Mild bronchiectasis was seen in several animals. Fibroblastic activity was observed in three days in animals which did not receive cortisone and was quite prominent in one animal which died in six days. In this animal numerous epithelium-covered processes filled with fibroblasts extended into the bronchiolar lumen. The epithelial layer over the polypoid mass was often attached at several points to the epithelium lining the wall of the bronchiole, forming "epithelial synechiae" (Fig. 9). In other bronchioles layers of reticulated epithelium lined small empty spaces among the fibroblasts in the lumen, giving a "recanalized" appearance (Fig. 10). Fibroblastic activity was not observed in cortisone-treated animals which died.

In areas of bronchiolar regeneration there were occasional mitotic figures and frequent small foci of transitional or squamous epithelium and irregular epithelial nests or buds, sometimes extending into surrounding acini. Bronchiolar regeneration was most prominent in satellite fashion around damaged bronchi. There was no evidence of epithelial regeneration directly from alveolar walls.

Animals Surviving. — Several animals which survived possessed essentially normal lungs, while others presented changes varying from mild interstitial thickening and focal scarring to well-defined bronchiolitis obliterans. Some animals, although ap-

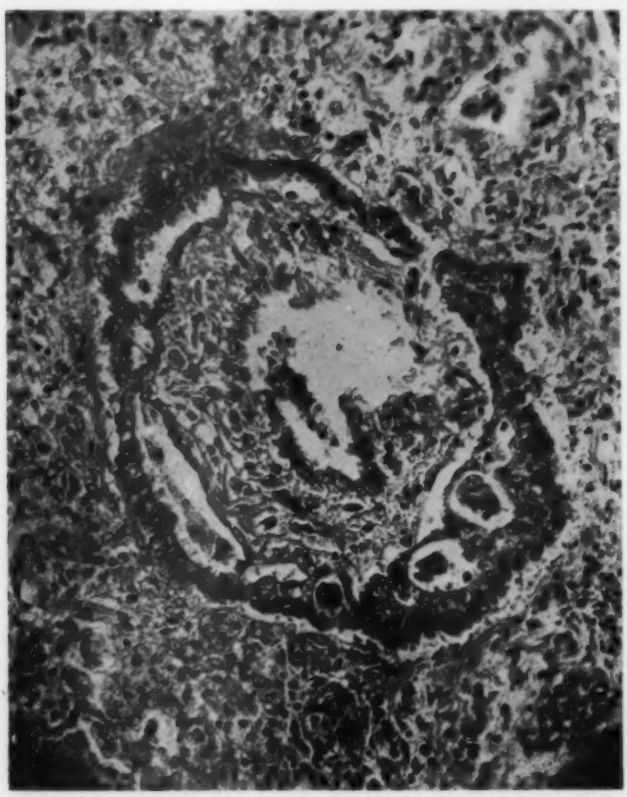

Fig. 10.—Bronchiolar plug made up of fibroblasts and regenerating epithelium with formation of epithelial-lined spaces. The appearance suggests "recanalization." Rabbit died in six days. Hematoxylin and eosin; × 280.

parently in good health, had focal pneumonic areas, bronchiectasis, focal bronchiolitis obliterans, or lung abscesses at autopsy.

No essential differences were found between the untreated animals and those given penicillin and streptomycin, and these will be considered together as the "noncortisone" animals. In the noncortisone group 13 of 26 rabbits (50%) developed focal bronchiolitis obliterans. These lesions varied in number and severity. Respiratory bronchioles were most commonly involved, but the larger bronchioles and occasional small bronchi were also affected. Grossly the lesions were seen as small, round or ovoid, gray or yellow nodules resembling miliary tubercles except for their irregular outlines and the fact that almost all of them on close inspection showed a central slit or opening representing the bronchiolar lumen. The lesions were most prominent in the lower portions of the lungs. Microscopically these lesions were similar to those previously described both in human and animal bronchiolitis obliterans. Some bronchioles contained polypoid projections, others showed partial obliteration of the lumens by scar tissue with only slit-like openings, and a few were completely obliterated. In most of the involved bronchioles the fibrous tissue extended into the bronchial wall and often into the surrounding lung parenchyma (Fig. 11). The involvement of surrounding parenchyma often originated in the respiratory bronchioles, from which the fibrous tissue extended out into the alveoli. Collagen deposition in involved areas was demonstrated by Masson trichrome stains in several animals killed in two weeks.

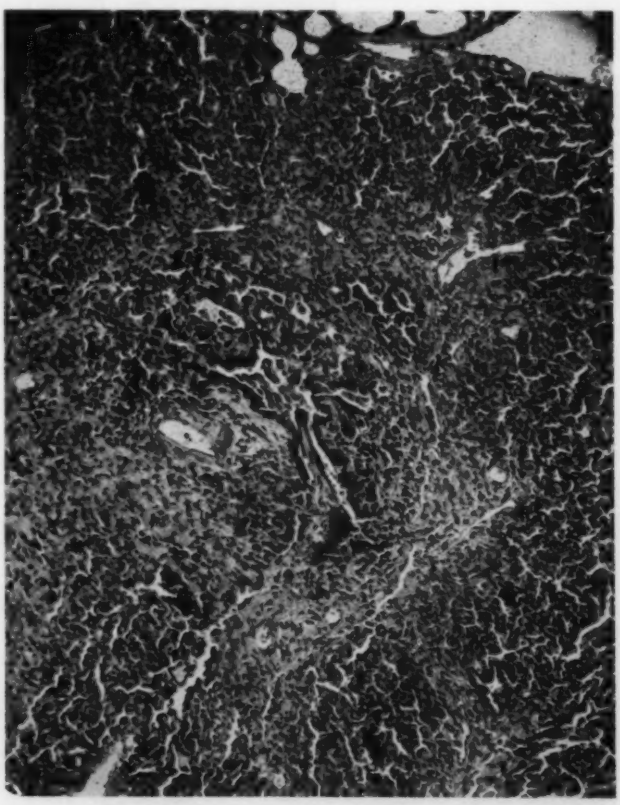

Fig. 11.—Distorted bronchiole entirely surrounded by scar tissue which extends into the parenchyma forming a fairly well-outlined nodule. Noncortisone rabbit two weeks after 1.0% nitric acid. Hematoxylin and eosin; × 140.

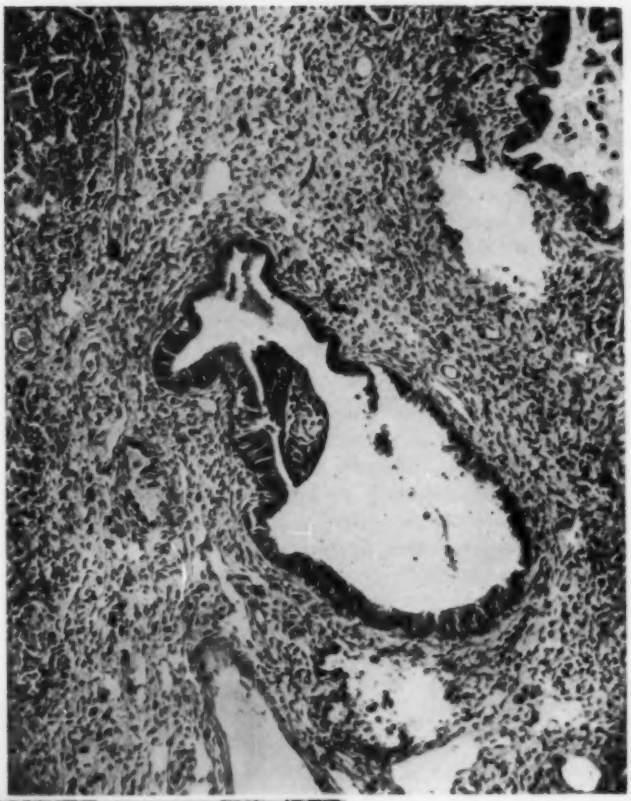

Fig. 12.—Bronchiolar epithelium-covered polyp and abundant scar tissue replacing most of the bronchiolar wall and the surrounding parenchyma. Noncortisone rabbit killed two weeks after intratracheal injection of 1.0% nitric acid. Hematoxylin and eosin; × 140.

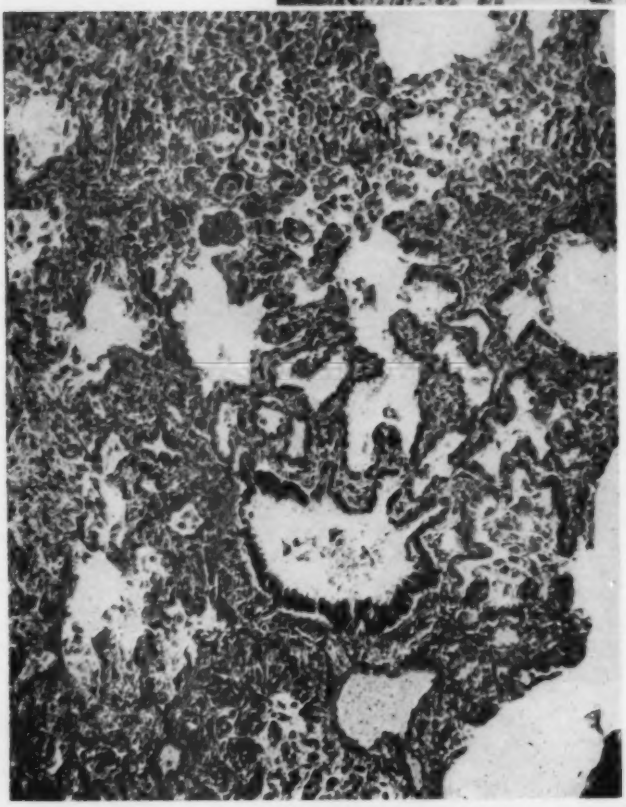

Fig. 13.—Cortisone-treated rabbit killed two weeks after intratracheal injection of 1.0% nitric acid, showing epithelial regeneration, slight emphysema, and mononuclear pneumonia but no fibrosis. Hematoxylin and eosin; × 140.

Bronchiolar regeneration was prominent. The polypoid projections were usually covered by epithelium, and at times several strands or ribbons of regenerating epithelium extended through the fibrous tissue, forming a reticulated pattern (Fig. 14). This epithelium was prominent around many of the damaged bronchi or bronchioles, often extending into and lining adjacent alveolar spaces. Alveolar walls away from the bronchioles did not show this change.

Changes in the cortisone-treated animals differed in several respects from the noncortisone animals. Fibrosis was markedly diminished, and epithelial regeneration was more prominent (Figs. 13 and 15). Pneumonia and abscess formation were slightly commoner. Definite bronchiolitis obliterans was present in only 1 of 12 animals in this group. In two other animals a few polypoid projections containing fibroblasts and covered by epithelium were seen, but no collagen was demonstrated by Masson stains prior to one month. In several animals killed at one month there was scanty collagen deposition in severely damaged areas but rarely involving recognizable bronchioles. The bronchiolar regeneration, possibly because of the absence of fibrous tissue, was much more prominent in the cortisone animals, although the epithelial changes had not seemed to progress in animals killed at two months, compared to those killed in two weeks.

Four of the twelve cortisone animals had small but grossly visible lung abscesses, compared to two of the twenty-six noncortisone animals. In three cortisone-treated animals large quantities of lipid material were present in the lungs. In paraffin sections this material was seen as varying-sized vacuoles. It was sudanophilic in frozen sections.

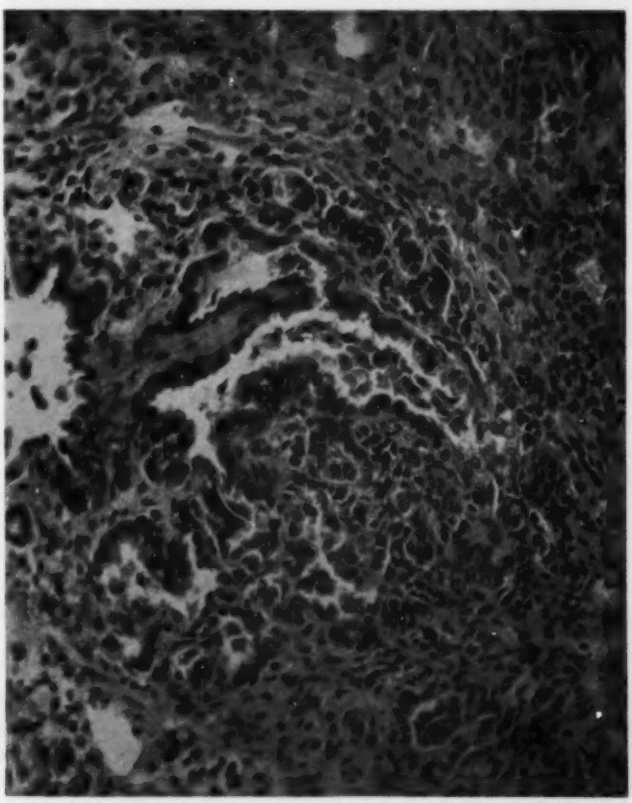

Fig. 14. — Noncortisone rabbit killed two weeks after intratracheal injection of 1.0% nitric acid, showing small polypoid bronchiolar projection, reticulated pattern of regenerated epithelium, and fibrous tissue. Hematoxylin and eosin; × 280.

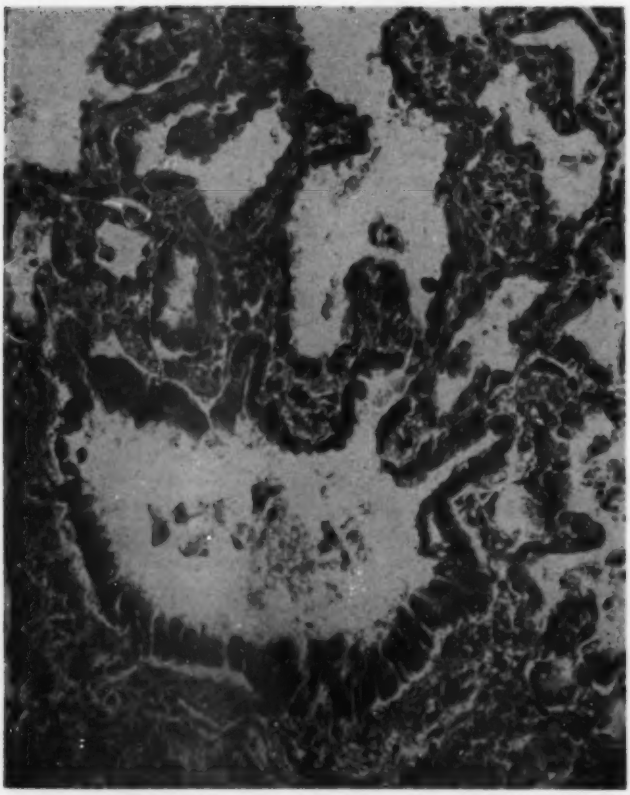

Fig. 15.—High power of Figure 13, from a cortisone-treated animal killed two weeks after injection. Epithelial regeneration is prominent, and the interstitial tissue is thickened and contains a few inflammatory cells, but there is no scar tissue. Hematoxylin and eosin; × 280.

Comment

Intratracheal injection of nitric acid is a simple, safe, and reliable method of producing focal bronchiolitis obliterans in rabbits. This technique provides a convenient means of evaluating therapy of the acute pulmonary edema and pneumonia produced by chemical agents, as well as of methods of preventing complications such as bronchiolitis obliterans.

Study at various time intervals of the animals which died of the intratracheal injections, as well as of those which survived, provided considerable information on the pathogenesis of the lesions produced by nitric acid. The first histologic change, demonstrable as early as two minutes, was hemorrhage in the capillaries of the respiratory bronchioles just beyond the epithelial lining. This predilection of site may account for the rather striking involvement of the small bronchioles, especially the respiratory units, in both human and experimental bronchiolitis obliterans following exposure to nitrogen dioxide. This involvement of respiratory bronchioles has been well illustrated by McAdams,[3] in his report of the death from bronchiolitis obliterans of a chemist exposed to fumes of nitrogen dioxide after an explosion of a flask containing red fuming nitric acid. Epithelial changes, also occurring first in the respiratory bronchioles, were evident in about two hours when the bronchiolar epithelium began to desquamate. In about 12 hours well-formed papillary processes made up of epithelium and lamina propria containing a coagulum of edema fluid, fibrin, and red blood cells were noted. These were especially prominent in respiratory bronchioles,

and the coagulum extended into the alveoli and larger bronchioles. Fibroblastic activity began in about three days, and well-formed scar was seen in two weeks.

The same time relationships or the same sites of initial damage apparently do not hold for bronchiolitis obliterans produced by other chemical agents. Winternitz [10] demonstrated, for example, that chlorine produced early severe damage to the upper respiratory tract, phosgene involved the small bronchi and bronchioles almost selectively, and chloropicrin occupied an intermediate position, producing less damage to the trachea and main bronchi than chlorine but more than phosgene. Chloropicrin and phosgene had similar effects on small bronchi and bronchioles. We have demonstrated previously that the polypoid projections can be produced very rapidly (two to three minutes) by intratracheal injection of ethyl alcohol.[13] However, aside from differences in the times and possibly in the initial sites of epithelial damage, the lesions of bronchiolitis obliterans produced by various chemical irritants are probably very similar in their development. The pathogenesis of bronchiolitis obliterans seen after influenza is also probably similar, since there is considerable epithelial damage very early in this disease and epithelial desquamation and papillary processes are common. Desquamation of the epithelium in almost all bronchioles and bronchi with formation of a few papillary processes were striking features in the lungs from a recent autopsy in our laboratory on a patient with proven Asian influenza pneumonia and no demonstrable bacterial infection.[20]

Epithelial regeneration from the reverse layer was seen as early as 20 hours. In one animal which died in 36 hours ciliated cells and cytoplasmic vacuoles were present in regenerated epithelium. The regenerating epithelium was seen in bronchioles and bronchi which still contained the desquamated damaged epithelium, but the presence of secretory vacuoles and cilia suggests that the regenerating epithelium was capable of its normal mature functions. We had not realized that complete epithelial regeneration occurred this rapidly in a chemically damaged bronchiole. Winternitz stated that epithelial regeneration was seen in dogs as early as 36 hours after insufflation of hydrochloric acid and that "in 52 hours many alveoli have a completely new layer of lining cells." [21] There was no direct evidence of an epithelial lining of alveolar spaces in the present study. As in a previous experiment,[18] the prominent bronchiolar regeneration in satellite fashion around damaged bronchi and bronchioles and the extension of regenerating epithelium from damaged respiratory bronchioles into nearby alveolar spaces suggested that all of the epithelium arises from bronchioles. Absence of regeneration in alveolar spaces away from the bronchioles indicates either that there is no epithelial lining in this area or that any epithelium present has lost its capacity to regenerate in response to chemical irritation.

Failure of combined penicillin and streptomycin to protect rabbits from the acute damage caused by nitric acid was expected, because death in the early stages occurred from acute pulmonary edema or pneumonia caused by the chemical changes rather than infection. The frequent finding of aspirated food may be explained by loss of cilia and possibly by loss of protective reflexes. This finding, also observed in previous experiments after injection of gastric juice or hydrochloric acid,[14] suggests that care must be used in the feeding of patients with respiratory diseases, especially those damaging the epithelium. The appearance of the sudanophilic material in damaged lungs differed considerably in the noncortisone and cortisone animals. The pathogenesis of these changes and the explanation of the differences between noncortisone and cortisone-treated animals are now being studied.

Prevention of the focal lesions of bronchiolitis obliterans in all but one animal treated with cortisone has both pathogenic and therapeutic implications. This finding along with the demonstration of the pro-

gression of the lesions at different time intervals supports our concept, developed during the study of the bronchiolitis obliterans produced by ethyl alcohol,[13] that the lesion results from the repair and scarring of the initially damaged area rather than from long-continued inflammation of the bronchial wall. The cortisone acts, apparently, by delaying fibroblastic activity until epithelial regeneration occurs in the damaged bronchioles and bronchi. Scar tissue eventually forms especially in portions of the lung where the epithelium has been completely destroyed by the initial chemical exposure.

The higher incidence of focal pneumonia and abscess formation in the cortisone-treated animals should not be used as an argument against the use of cortisone in human patients after exposure to chemical irritants known to produce bronchiolitis obliterans. The patient after exposure to chemical irritation can be watched more carefully for signs of secondary infection, and a variety of antibiotic agents are available for control of secondary bacterial infection. In this experiment cortisone was often continued until the animals were killed, and the dosage employed was probably higher than that required for human use. In several animals in which cortisone was used only for four or five days, bronchiolitis obliterans was not found, suggesting that the cortisone may be needed to delay fibroblastic activity only until epithelial regeneration occurs. Since we have shown that regeneration of morphologically mature epithelium occurs under the conditions of this experiment in 24 to 36 hours, cortisone may be required for only a few days.

The results suggest that to obtain maximum effectiveness in the therapy of human bronchiolitis obliterans, cortisone should be started as soon after the initial damage as possible, without waiting until fibrosis had already occurred. This concept is especially applicable in patients with a definite history of exposure to chemical irritants, including the nitrogen oxides, in whom a fairly high percentage of those surviving the acute phase might be expected to develop bronchiolitis obliterans. However, application of these results to prevention of human bronchiolitis obliterans must be made cautiously, since it is known that cortisone delays fibrosis in the rabbit much more effectively than in such species as the guinea pig, monkey, and man.

Summary

Intratracheal injection of a dilute solution of nitric acid provides a simple and convenient method of producing bronchiolitis obliterans. Focal bronchiolitis obliterans was produced in 13 of 26 rabbits (50%) surviving intratracheal injections of 1.0% or 1.2% nitric acid. The pathogenesis of bronchiolitis obliterans produced by this method has been traced. The first demonstrable change is hemorrhage from the capillaries of the respiratory bronchioles. This is followed by necrosis and desquamation of the bronchiolar epithelium, with formation of coagulum-filled epithelium-covered papillary processes in about 12 hours, fibroblastic activity in about 3 days, and definite scar tissue in 2 weeks. Regeneration of morphologically differentiated bronchiolar epithelium is present in some animals in 24 to 36 hours.

The use of combinations of penicillin and streptomycin or cortisone, penicillin, and streptomycin did not affect the survival rate of the animals, approximately two-thirds of which died from acute pulmonary edema or chemical pneumonia. A combination of penicillin and streptomycin did not influence the development of bronchiolitis obliterans in surviving animals, but a combination of cortisone, penicillin, and streptomycin prevented bronchiolitis obliterans in all but one of 12 surviving rabbits. The cortisone administration was begun the day prior to the injury.

The effect of cortisone in preventing bronchiolitis obliterans and the demonstration of the progression of the lesions at various time intervals support the concept that bronchiolitis obliterans develops from repair by fibrosis of the initial damage to the

bronchiolar epithelium rather than from long-continued inflammation of the bronchiolar wall. Therapeutic implications of the use of cortisone, especially its immediate employment, in human beings exposed to chemical irritants known to produce bronchiolitis obliterans are discussed.

Two University of Pittsburgh medical students, Mr. William Stept and Mr. Edward O'Donnell, provided technical assistance, and Mr. Albert Levin, University of Pittsburgh Medical Photographer, did the photography.

Director of Laboratories, Presbyterian Hospital, 230 Lothrop St. (13).

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Hemorrhagic States Secondary to Intravascular Clotting

An Experimental Study of Their Evolution and Prevention

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Ordinarily blood coagulation serves to prevent bleeding. At times, however, widespread intravascular clotting occurs and appears to cause a hemorrhagic state.[1,2] Conditions in which such antipodal disturbances in the hemostatic mechanism might be seen include hemorrhagic complications of pregnancy, surgical trauma, burns, shock, transfusion reactions, and neoplasia. Earlier work from our laboratory suggests that similar systemic reactions may take place when tissue necrosis is induced by exposure to cold.[3] More recently, the generalized Shwartzman phenomenon has also been regarded as belonging to this group of hemorrhagic disorders.[4] Several pathogenic mechanisms have been suggested to explain the paradoxical sequence of clotting followed by hemorrhage. One proposal is that tissue products with thromboplastic activity gain access to the blood stream and accelerate intravascular coagulation; deficiencies of clotting factors result and produce a bleeding tendency. The factors that are diminished appear to be those known to be consumed during coagulation.

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During in vitro clotting, the consumption of clotting factors is impaired unless there is an intact system. If antihemophilic factor (AHF) is lacking, prothrombin and fibrinogen conversion are retarded.[5] If thrombin sources are lacking, AHF does not disappear.[6] This investigation was undertaken to determine if animals with comparably defective clotting systems would be protected from the effects of intravenously administered thromboplastin. Three types of dogs in which there was defective coagulation were studied: (1) hemophilic dogs, (2) dogs treated with bishydroxycoumarin (Dicumarol), and (3) dogs in whom liver injury was induced by chloroform administration. The blood of the first type of animal is naturally deficient in AHF; that of the other two types is deficient in prothrombin and other factors concerned with thrombin evolution. The responses of these animals to injected thromboplastin were carefully compared to those of normal control dogs. In these experiments, two different types of thromboplastin were administered: (1) crude tissue extracts, which are regarded as "complete" thromboplastins, and (2) ultracentrifuged preparations, which are regarded as "partial" thromboplastins.[7] Preliminary reports of some of these data have been made previously.[8,9]

Materials and Methods

Adult hemophilic dogs were obtained from our colony.[10] All other dogs were mongrel animals of comparable weight, selected without regard to sex. Dogs were chloroformed by a previously used

procedure.[11] The dogs treated with bishydroxycoumarin were given an oral preparation sufficient to prolong the prothrombin time to 30 seconds or more. Crude and ultracentrifuged dog lung thromboplastin preparations were made by methods previously described[7] and were stored at —20 C until used. Different preparations had varied activity in the "prothrombin time" test[12]: recalcified dog plasma clotted in 7-9 seconds with complete thromboplastin and in 16-18 seconds with partial thromboplastin. "Heated thromboplastin" was prepared by heating the ultracentrifuged material at 100 C for 30 minutes; after heating, it was devoid of thromboplastic properties. In all experiments, thromboplastin was rapidly injected in a period of three to five seconds into the external jugular veins in doses indicated in the figure legends. Before injection, and at various intervals thereafter, blood samples were removed and used for determinations of "prothrombin time," [13] fibrinogen,[13] platelets,[14] prothrombin,[11] and AHF.[7] Values for normal dogs by these methods were as follows: prothrombin time, 6.0-9.0 seconds; fibrinogen, 250-350 mg. per 100 ml.; platelets, 200,000 to 400,000 per cubic millimeter. Both prothrombin and AHF were measured in terms of per cent activity of a known normal sample. Results are expressed in per cent of preinjection control values.

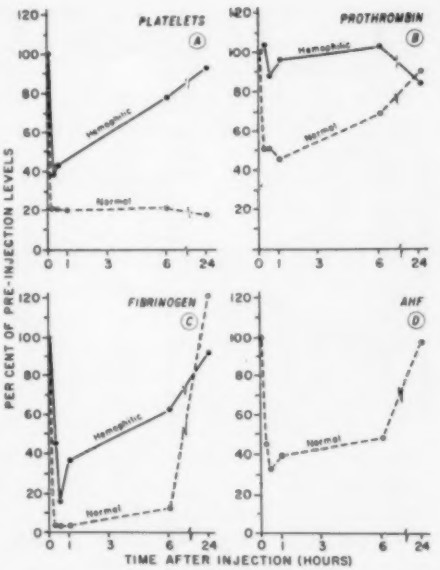

Fig. 1.—Response of a normal male dog (15 kg.) and a hemophilic male dog (15 kg.) to 2 ml. complete thromboplastin diluted to 10 ml. with isotonic saline.

Results

Effect of AHF Deficiency on Response to Circulating Thromboplastin.—The response to potent or "complete" thromboplastic preparations was studied first. Two normal and two hemophilic dogs were given injections. Figure 1 illustrates the response of each type of dog. Within 15 to 20 seconds all animals exhibited minor convulsive movements, hyperpnea, cyanosis, nystagmus, and excessive salivation. However, the reaction in the hemophilic dogs was not as severe or prolonged as in the normal animals. All dogs exhibited thrombocytopenia, as illustrated in Figure 1*A*. In this experiment the platelet depression in the normal animal was protracted for the 24-hour experimental period, whereas the platelet count of the hemophilic animal returned toward the preinjection value during this time. Prothrombin concentration (Fig. 1*B*) in the normal dog fell to 45% after the injection; the prothrombin concentration in the hemophilic animal was not appreciably affected. Hypofibrinogenemia was observed in both types of dogs (Fig. 1*C*), but the decrease in the normal was more pronounced and more prolonged than in the hemophilic dog. Fibrinogen concentrations in both dogs returned to normal limits within 24 hours. The AHF level in the normal dog dropped abruptly to 33% of control levels and then gradually rose to preinjection levels during the next day.

The response to partial thromboplastin is illustrated in Figure 2. The normal dog showed signs of respiratory difficulty, hyperkinesis, excessive salivation, and tachycardia after thromboplastin injection. The hemophilic dog showed no apparent signs of response. Thrombocytopenia rapidly developed in the normal animal and persisted throughout the experiment. The minor variations in platelet values noted in the hemophilic dog were within the limits of the method employed. Prothrombin concentration was not appreciably altered in either animal. The fibrinogen level in the

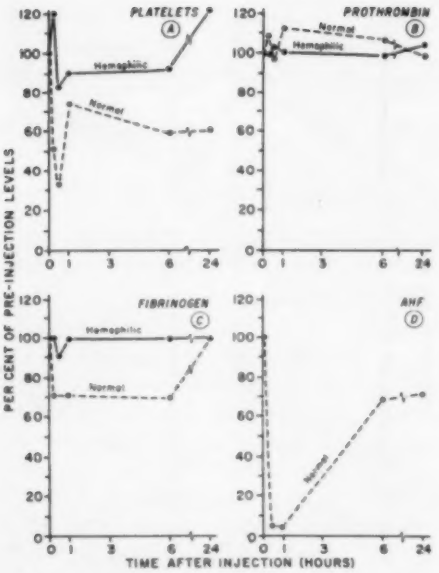

Fig. 2.—Response of a normal female dog (20 kg.) and a hemophilic male dog (15 kg.) to 0.4 ml. partial thromboplastin diluted to 10 ml. with isotonic saline.

normal dog decreased to 70% 15 minutes after thromboplastin injection and returned to normal levels within 24 hours. Fibrinogen concentration in the hemophilic dog, on the other hand, was never significantly altered. AHF depression again occurred in the normal dog, with the level falling to 5% in 15 minutes and returning toward normal limits during the next 24 hours. Results of the prothrombin time test on plasma of these dogs are recorded in Table 1. The response of the prothrombin time to the injection of 2-4 ml. of other partial thromboplastin preparations was determined on four other normal dogs. Thromboplastin injection prolonged the prothrombin time of the plasma in each instance. In contrast to the normal animals, no changes occurred in the hemophilic dog.

Effect of Bishydroxycoumarin on Response to Circulating Thromboplastin.—Partial thromboplastin was injected into each of four dogs treated with bishydroxycoumarin and two normal dogs. Both normal animals developed respiratory difficulty,

TABLE 1.—*Effect of Injecting Partial Thromboplastin on "Prothrombin Times" of Normal and Hemophilic Dogs*

Experimental Animal	Time After Thromboplastin Injection, Hr.					
	0	¼	½	1	6	24
	Prothrombin Time, Sec. *					
Normal dog	7.3	10.6	22.5	10.4	9.2	5.9
Hemophilic dog	6.0	6.7	5.9	6.2	5.2	6.8

* Each dog received 0.4 ml. of the same thromboplastin suspension.

hyperkinesis, tachycardia, and pupillary dilation; in addition, micturition, defecation, and mucosal petechiae occurred in one dog. Contrastingly, the animals treated with bishydroxycoumarin showed no overt response to the injection. The responses of clotting factors in a typical experiment are shown in Figure 3. In the normal dog, transient thrombocytopenia, hypoprothrombinemia, hypofibrinogenemia, and secondary hemophilia evidenced a severe reaction to this thromboplastin preparation. In the animal treated with bishydroxycoumarin,

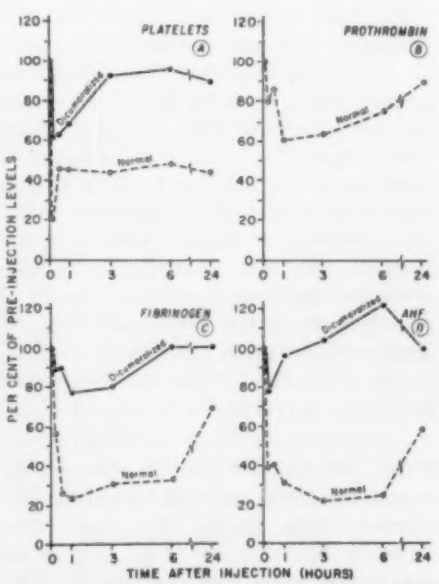

Fig. 3.—Response of a normal male dog (20.5 kg.) and a male dog (15 kg.) treated with bishydroxycoumarin to 4 ml. of partial thromboplastin, diluted to 10 ml. with isotonic saline.

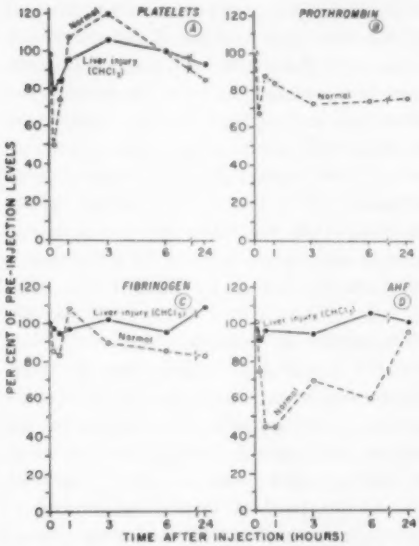

Fig. 4.—Response of a normal male dog (14.9 kg.) to 2 ml. of partial thromboplastin and a chloroformed male dog (22.0 kg.) to 12 ml. of partial thromboplastin.

the changes were much less pronounced; the prothrombin level in this dog was already depressed to 15 units per milliliter prior to injection of the thromboplastin.

Effect of Liver Injury (Chloroform Treatment) on Response to Circulating Thromboplastin.—Partial thromboplastin was injected into four dogs to whom chloroform had been administered by inhalation 48 hours earlier. Two normal control dogs were simultaneously studied. The normal animals again showed reaction signs similar to those described previously. The chloroform-treated animals, even when given up to six times as much thromboplastin as the normal dog, showed no apparent response. Figure 4 is illustrative of the results. As would be expected, chloroform administration alone had produced low levels of prothrombin (16 units per milliliter) and fibrinogen (66 mg. per 100 ml.). Unexpectedly, it also depressed AHF values in all four dogs to about 45% of the levels before chloroform treatment (range, 32%-58%). As in the other figures, however, the preinjection levels have been assigned a value of 100%, and so the relative response to thromboplastin can be compared. A transient platelet depression occurred in the normal animal after thromboplastin injection, but within one hour the platelet count returned to normal limits; the chloroformed dog did not develop significant thrombocytopenia. A slight reduction in prothrombin concentration was observed in the normal dog 15 minutes after thromboplastin was given. Fibrinogen levels were not strikingly affected in either the control or the chloroformed dogs by the injections. A decrease in the AHF level of the normal animal was rapidly effected by the intravenous thromboplastin injection, but the sixfold larger dosage of thromboplastin produced no significant change in the AHF levels of the chloroformed dog.

Response to Heated Thromboplastin.—An experiment was conducted to determine if the systemic effects of thromboplastin could be correlated with its clot-promoting properties. An active extract was first shown to have an effect by injection of 2 ml. of partial thromboplastin into a normal

TABLE 2.—*Relative Effects of Active and Heat-Inactivated Thromboplastin in Normal Dogs* *

Time After Injection, Min.	Active Thromboplastin			Heat-Inactivated Thromboplastin		
	Platelets, %	AHF, %	"Prothrombin Time," Sec.	Platelets, %	AHF, %	"Prothrombin Time," Sec.
0	100	100	9.0	100	100	6.4
10	50	74	12.3	107	125	6.0
30	74	44	12.0	—	—	—
60	107	44	12.0	101	90	6.0

* Both dogs were males and weighed 14.9 and 15.2 kg., respectively; 2 ml. of ultracentrifuged thromboplastin was injected.

dog and observation of signs similar to those already described, as well as the development of thrombocytopenia, depression of AHF, and prolongation of the prothrombin time. After the thromboplastin was inactivated by heating, 2 ml. was injected into another normal dog. The responses of both dogs are indicated in Table 2. The dog that received the heated thromboplastin showed no significant depression in the levels of his clotting factors and no apparent signs of reaction.

Comment

Following intravascular injection of tissue thromboplastin, normal dogs exhibited severe reactions and striking changes in the concentrations of blood factors. Apparently these reactions were related to intravascular coagulation. Among the responses observed in the normal dogs were hypoprothrombinemia, hypofibrinogenemia, thrombocytopenia, AHF depression, and prolongation of the prothrombin time. The dogs also developed transitory shortening of the Lee-White clotting times, followed by a phase of prolongation. Such a "positive phase" and "negative phase" have been observed since the time of the earliest studies on induced intravascular clotting.[15] Most of the responses observed in our experiments were roughly related to the amount and activity of the thromboplastin administered. A spectrum of reaction can be produced in the normal dog, depending on the dosage and potency of thromboplastin used and the speed of administration. It was therefore necessary to control each reaction to thromboplastin by a simultaneous injection into a normal animal. The most consistent responses in our experiments, regardless of the thromboplastin used, were depressions of the levels of AHF and platelets and prolongation of the prothrombin times.

In striking contrast to the reaction in normal dogs, hemophilic dogs, or dogs with deficiencies of clotting factors induced by treatment with bishydroxycoumarin or chloroform responded much less dramatically or not at all to injected thromboplastin. Apparently these deficiency states were sufficient to retard intravascular coagulation and consumption of clotting factors. The chloroform-treated animals were the most resistant and were able to tolerate large amounts of active tissue thromboplastic materials. On the other hand, it was possible to overcome partially the resistance of the hemophilic dogs by using crude tissue extracts. This is in keeping with in vitro observations of Langdell and associates[7] that the accelerated clotting time of hemophilic plasma is equal to that of normal plasma when complete thromboplastins are used but prolonged if partial thromboplastins or low concentrations of crude thromboplastins are used. Conceivably, dilution of the crude material in the blood stream would explain why the response of the hemophilic dog to crude thromboplastin was less pronounced than that of the normal dog.

Depression of AHF levels following experimental injections of thromboplastic substances has been previously observed by us[5] and by others.[16] Similar AHF changes have been noted in a human patient with premature separation of the placenta.[17] It is of interest that thrombin inactivates AHF during extravascular coagulation.[6] Perhaps the same thrombin-inactivating mechanism operates during intravascular coagulation to lower the antihemophilic activity of blood. Such an explanation is supported by our observations that no significant AHF loss occurred when the thrombin evolution that ordinarily follows thromboplastin injection was suppressed by the administration of bishydroxycoumarin or chloroform.

The thrombocytopenic response has been widely observed in many conditions thought to be associated with intravascular clotting, and some investigators have suggested that it depends primarily on the particulate nature of material injected into the blood stream.[18] This was not our experience, since heat inactivation of thromboplastic suspensions abolished the thrombocytopenic effect.

Rather, thrombocytopenia appeared to be more closely correlated with reduction in fibrinogen levels.

The prothrombin time seems to be a sensitive indicator of response to circulating thromboplastin. It was prolonged in all the normal control dogs after tissue thromboplastin had been injected, even when the two-stage prothrombin or fibrinogen determinations showed no measurable change. This prolongation may reflect either inhibitor elaboration or decreases in accelerator factors known to influence the one-stage prothrombin test.

During the course of these experiments we were unable to demonstrate consistently activation of profibrinolysin, a mechanism thought by some to account for the hemorrhagic complications of pregnancy and related clinical diseases.[19] On the contrary, our findings are consistent with the hypothesis that intravascular coagulation and resultant consumption of clotting factors is the basic mechanism leading to a bleeding tendency. Such a hypothesis explains the paradox of a hemorrhagic tendency being prevented by the prior establishment of a hypocoagulable state.

In contrast to a previous report,[20] our experiments indicate a significant depression of antihemophilic factor in dogs after exposure to chloroform. This did not occur after treatment with bishydroxycoumarin. To explain this observation, further studies are needed; possibly the liver is the site of AHF production. Previous work has suggested that the presence of necrotic tissue is associated with AHF depression.[3] It might be that the effect of chloroform on AHF is related to necrosis of liver cells and release of thromboplastic substances.

Summary and Conclusions

Thromboplastic tissue extracts were injected into normal dogs, hemophilic dogs, and dogs treated with bishydroxycoumarin (Dicumarol) and with chloroform. The normal dogs exhibited a characteristic reaction pattern of neuromuscular and cardiorespiratory disturbances. In addition, they developed thrombocytopenia, hypoprothrombinemia, hypofibrinogenemia, and depressions in the antihemophilic activity of their plasma. These responses were diminished or abolished in dogs with hypocoagulable blood. Three types of animals with defective clotting mechanisms were studied: hemophilic, bishydroxycoumarin-treated, and chloroform-treated dogs; each was resistant to thromboplastin injection.

These findings indicate similarity between the response to injected thromboplastin and reactions known to occur during in vitro coagulation. They tend to support the concept that the hemorrhagic states in conditions associated with circulating thromboplastic tissue products probably result from widespread intravascular coagulation and concomitant consumption and depression of many clotting factors.

Acute liver necrosis, as produced by exposure of four dogs to chloroform inhalation, lowered the antihemophilic factor levels to about 45% of their original values.

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The Prevention of the Generalized Shwartzman Reaction by Fibrinolytic Activity

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The generalized Shwartzman reaction is induced in rabbits by the intravenous injection of two doses of bacterial endotoxin. After the first dose, intravascular thrombi are found in the capillaries of the lung, liver, and spleen. After the second injection, thrombi appear in increased numbers in these organs and in the kidney as well. The renal glomerular thrombi lead to ischemic necrosis of the renal cortices, bilateral cortical necrosis, which has been regarded as the hallmark of the generalized Shwartzman reaction.

Recent studies have indicated that this reaction is basically due to the effect of the endotoxins on the blood coagulation system.[1-3] The circulating fibrinogen decreases markedly at the time the thrombi are forming. The decrease in fibrinogen is due to the utilization of this substance in the thrombotic process, since no fibrinolytic or fibrinogenolytic activity occurs during the reaction.

The generalized Shwartzman reaction is completely prevented by heparin treatment of the animals prior to administration of the toxin.[4,5] It is also prevented by prior administration of warfarin sodium. The major effect of heparin is to prevent the action of thrombin on fibrinogen but it also acts as an anti-thromboplastic agent. One of the effects of warfarin sodium is to reduce the amount of prothrombin complex in the circulating blood. Thus two anticoagulants acting at different points in the blood coagulation mechanism are capable of preventing the Shwartzman reaction.

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Another way to attack the problem of intravascular thrombosis is by way of fibrinolytic activity. Tillett and Garner[6] showed that streptococci elaborate a substance capable of destroying already formed fibrin, a reaction later shown to be mediated by plasmin.[7] Since then streptokinase preparations have been widely used to destroy unwanted collections of fibrin. More recently, a number of authors have investigated the intravascular use of streptokinase preparations in the hope of eradicating intravascular thrombi after their formation.[8-13] Some success in this area has been claimed in the human beings, and several workers have shown that plasmin and plasminogen activators can rapidly lyse large arterial and venous thrombi.

Therefore, the question arose as to whether or not the artificial activation of fibrinolytic activity by streptokinase during the devlopment of the generalized Shwartzman reaction would destroy the clots that form in the capillaries, precapillary arterioles, and venules in this reaction. During the course of these experiments the work of Condie et al.[14] on the reversal of the generalized Shwartzman reaction by streptokinase appeared. Their studies show that the activation of fibrinolytic activity by streptokinase does indeed prevent this reaction.

It is the purpose of this report not only to confirm their work but in addition to show that while fibrinolytic activity prevents the deposition of thrombi, it is incapable of destroying preformed thrombi after a 24-hour period.

Materials and Methods

Hybrid weanling albino rabbits weighing approximately 1 kg. were used in these experiments.

They were prepared with an intravenous injection of 0.2 mg. of Shear's polysaccharide * and challenged 24 hours later with a second intravenous injection of 0.2 mg. of polysaccharide. One group (Series A) of 10 animals was treated in this manner and served as the control group.

A second group, Series B, of 11 animals, comprised the test group, which was treated in exactly the same manner with the exception that at 30 minutes after the second injection of toxin they were given streptokinase-streptodornase (Varidase †) intravenously. The streptokinase was given at the rate of 50,000 units every 30 minutes until a clot lysis time of less than 30 minutes was observed. Blood was drawn from the marginal ear vein, allowed to clot in a glass tube, and observed at room temperature for the time of dissolution of the clot. The end-point was considered to be the time when 75%-100% of the clot was dissolved. Most animals required 100,000 units of streptokinase to achieve this point, and none required more than 150,000 units. The animals were killed 24 hours after the second injection of endotoxin by the intravenous injection of air. A complete autopsy was performed on every animal, and sections of lungs, kidneys, spleen, and liver were examined microscopically after staining with hematoxylin and eosin.

Another control group of 10 animals was given streptokinase only and then examined pathologically to eliminate the possibility that this material, derived from bacterial cells, might have the ability to induce thrombosis. By serial determinations of the clot lysis time on these animals, it was observed that the fibrinolytic activity alone did not obviate clotting and that fibrinolytic activity persisted for at least three hours after first being observed.

A second test group of nine animals were given a single injection of Shear's polysaccharide (0.2 mg.) followed in 30 minutes by 100,000 to 150,000 units of streptokinase. These animals were killed 24 hours after the injection of endotoxin and examined pathologically in the same manner as the preceding groups.

* Dr. Murray J. Shear, of the National Institutes of Health, Bethesda, Md., furnished the polysaccharide.

† Lederle Laboratories Division, American Cyanamid Company.

TABLE 1.—*Extent and Location of Thrombi in the Generalized Shwartzman Reaction in Ten Animals*

	Lungs	Liver	Spleen	Kidney	
1+	4	0	0	1	
2+	0	5	0	0	
3+	0	3	4	0	
4+	1	0	1	4	(Bilateral renal cortical necrosis)
Total	5	8	5	5	

Results

The location and extent of intravascular thrombosis in the 10 animals given two doses of endotoxin are presented in Table 1.

The semiquantitative "0 to 4+" indicates the relative number of thrombi in each organ: 1+ indicates one thrombus in the section examined; 2+ equals many capillary and venular thrombi; 3+ equals ½ to ¾ of all capillaries occluded by thrombi, and 4+ equals virtually all small vessels filled with thrombi. The number of organs in which thrombi were found is of roughly the same order as in our previous experiments [1] with use of the same endotoxin. Five of these animals had bilateral renal cortical necrosis.

TABLE 2.—*Extent and Location of Thrombi After Two Doses of Endotoxin Plus Streptokinase in Eleven Animals*

	Lungs	Liver	Spleen	Kidney
1+	6	3	0	1
2+	1	1	0	0
3+	0	2	2	0
4+	0	1	0	0
Total	7	7	2	1

The extent and location of thrombi in the group of animals receiving two doses of toxin accompanied by streptokinase 30 minutes after the second injection are presented in Table 2.

The use of streptokinase after the second injection of endotoxin completely prevented the appearance of bilateral renal cortical necrosis. However, it can be seen that there was little or no evidence of reduction in the extent of thrombosis in the lungs, liver, and spleen when compared with the control series.

Since thrombi appear in these organs after the first injection, another test group was studied with use of streptokinase after the first injection of endotoxin to see whether

TABLE 3.—*Extent and Location of Thrombi After One Dose of Endotoxin in Thirty-Seven Animals*

	Lungs	Liver	Spleen	Kidney
1+	8	4	5	4
2+	6	2	10	1
3+	0	3	5	1
4+	0	0	1	0
Total	14 (38%)	9 (24%)	21 (57%)	6 (16%)

or not thrombosis in these locations could be prevented by fibrinolysis. The distribution and extent of thrombi in a control group after one injection of toxin are presented in Table 3 (from McKay and Shapiro [1]).

The distribution and extent of thrombi after one injection of endotoxin followed in 30 minutes by streptokinase are presented in Table 4.

These data indicate the great reduction in the number of thrombi in the liver, spleen, and lungs when streptokinase is given with the first dose of toxin.

The animals that received streptokinase alone showed no thrombi, nor did they show any morphologic alteration which could be attributed to streptokinase administration.

Interpretation

These experiments indicate that thrombi either will not form or are greatly reduced in number when fibrinolytic activity is present during the time when they usually form (one to four hours after the intravenous injection of bacterial endotoxin). This, coupled with the fact that no necrosis or hemorrhage can be found in the absence of thrombi, contributes to an understanding of the basic mechanism of the generalized Shwartzman reaction. The occurrence of thrombi in this reaction has long been known.[15] The identity of the substance which comprises these intravascular thrombi, however, has been a matter of some discussion. Booth et al.[16] have suggested that the material is derived from break-down products of blood vessel walls which were presumably damaged by exposure to the toxin. It will be noted, however, that no damage occurs to the blood vessel walls when the animals have been treated with fibrinolysin activator or with the anticoagulants heparin and warfarin. Therefore, it is clear that unless thrombosis occurs, no vessel necrosis is produced, even though the usual amounts of toxin are present in the circulating blood.

The prevention of thrombi by fibrinolytic activity indicates that from the standpoint of occlusion of the lumens of the small vessels, the single most important constituent of the thrombi is *fibrinogen* (fibrin). This does not mean that other substances may not be present in the thrombi. One would expect, for example, to find small amounts of serum albumin and globulin and perhaps a small amount of endotoxin. Indeed, the histochemical studies of Booth [16] suggest the presence of many substances found in the normal circulating blood—i. e., neutral fats, fatty acids, cholesterol and its esters, phospholipids, and glycoprotein. These substances are probably "occluded" in the thrombi which form in the circulating blood and do not have the primary significance of fibrinogen in the pathogenesis of this reaction.

The prevention of the thrombi by fibrinolysis is another piece of evidence that fibrinogen (fibrin) is the major constituent of the thrombi.

These experiments in addition to those using warfarin and heparin lay to rest any notion that a direct toxic action is responsible for hemorrhage and necrosis in the generalized Shwartzman reaction.

This experiment also sheds a little light on the effects of fibrinolysis. It was noted that there was no effect of fibrinolysis on the thrombi which form with the first injection of toxin in the liver, lungs, and

TABLE 4.—*Extent and Location of Thrombi After One Injection of Toxin Plus Streptokinase in Nine Animals*

	Lungs	Liver	Spleen	Kidney
1+	0	1	0	0
2+	0	0	3	0
3+	0	0	0	0
4+	0	0	0	0
Total	0	1 (11%)	3 (33%)	0

spleen when the streptokinase was administered with the second dose of toxin. (This, in spite of the fact that the thrombi that ordinarily form with the second injection in the kidney did not appear.) The liver, lung, and spleen thrombosis was prevented or reduced when the streptokinase was given with the first injection of toxin. These findings indicate that the *fibrinolytic activity must be present during the period when the thrombi are forming* in order to exert its lytic effect. Fibrinolysis is ineffective against the small vessel thrombi that have been present for 24 hours. This differs from the experience of several authors with the effect of fibrinolysis on large arterial and venous thrombi, where it is claimed that thrombi as old as three days can be readily lysed.[12] The microthrombi of the Shwartzman reaction must differ in this respect from the macroscopic intravascular thrombi produced by Johnson, Cliffton, and Back.[8,11,12] This may in part be explained by the fact that the tiny thrombi in the spleen and lung occlude the lumens of the small vessels and the circulation is completely stopped in those vessels. Therefore, the fibrinolytic activity in the circulating blood has no access to these clots except at their proximal ends. However, in the case of the liver thrombi this cannot be the explanation. These are often mural thrombi in a central vein which still has an otherwise widely patent lumen and an obviously good flow of blood. It would seem that these mural thrombi had an adequate exposure to fibrinolytic enzyme but somehow resisted its effect. Perhaps the greater density and compactness of the fibrin masses in this reaction as compared with the widely separated, loosely knit, thin strands of fibrin in a macroscopic thrombus accounts for this difference of response to the proteolytic enzyme.

The suggestion of Condie[14] that the Shwartzman reaction is "reversed" by fibrinolytic activity must be considered in the light of these findings. Since the fibrinolytic activity must be present during the period when the thrombi are deposited, it may be more precise to use the word "prevention" rather than "reversal."

It should be noted that an alternative explantion for this effect of fibrinolysin might be that the enzyme destroyed all the circulating fibrinogen, thereby removing the possibility of thrombus formation. However, this is an unlikely explanation, since Johnson and Tillett demonstrated that streptokinase-induced fibrinolysis does not completely remove the circulating fibrinogen even though it lowers the fibrinogen level.[8] A few of our own determinations showed that animals did not have afibrinogenemia or even any great lowering of the fibrinogen level as a result of streptokinase injection.

Summary

The injection of streptokinase with the second injection of bacterial endotoxin prevents the development of renal thrombi and bilateral renal cortical necrosis of the generalized Shwartzman reaction.

The injection of streptokinase with the first injection of bacterial endotoxin greatly reduces the number of thrombi in the liver and lungs.

Fibrinolytic activity does not destroy microscopic thrombi in this reaction when they have been present for 24 hours.

These observations suggest that in order for the fibrinolytic enzyme to destroy these microscopic thrombi it must be "occluded" in the thrombi, and they are further evidence that the thrombi of the generalized Shwartzman reaction are composed of fibrin.

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The Cytotoxic Action of Normal Human Serum on Certain Human Cells Propagated in Vitro

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Recent investigations in the field of immunochemistry have related the virus-neutralizing, bacterocidal, and parasitocidal activity of normal serum to the complement-properdin system.[1] Other investigations have been concerned with a possible relationship of this system to neoplastic disease.[2-4] It was observed in this laboratory that normal human sera were rapidly lethal to certain strains of human cells propagated in vitro. The purpose of this investigation was to explore the nature and significance of the observed cytotoxic action of normal human serum in relation to the complement-properdin system.

General Methods and Materials

A. Cell Cultures and Media.—All cells were propagated in Medium 29, according to techniques described by Swim and Parker.[5,6,11,12] Medium 29 contains 20% normal horse serum and 80% S-103,[6] which is composed of 20 amino acids, 8 vitamins, and the ingredients of Earle's saline.[6]

Strain U12-29: This cell is a fibroblast derived from human uterus, which has undergone transformation in vitro so that morphologically and physiologically it closely resembles a malignant cell.[7,8] Strain U12-29 is a nutritional variant isolated from a culture of U12-705 and is distinguished by the fact that chick embryo extract is not required for growth.[6] The U12 cells typically grow as incoherent, stellate, fusiform, or triangular forms, extending short thin cytoplasmic processes. The nuclei are large and round, with coarsely granular chromatin. Mitotic figures are common (Fig. 1A).

Strain HeLa 29: Strain HeLa was derived originally from an epidermoid carcinoma of the uterine cervix.[9] HeLa 29 is derived from a strain which has been propagated serially in media containing normal horse serum in place of human serum for at least a year.[10] The cells employed in these experiments were from generations 39 through 64. The HeLa 29 cell closely resembles the U12 morphologically (Fig. 2A).

Strain FS: This is a newly established strain of human fibroblasts derived from newborn foreskin, according to the method of Swim and Parker.[11] The cells used in these experiments were from the first five generations in vitro. In contrast to the permanent lines described above, these newly established cultures contain relatively normal-appearing fibroblasts, which are densely packed coherent bundles of bipolar spindle-shaped forms with very long cytoplasmic processes and ovoid nuclei (Fig. 3).

*B. Serum and Serum Reagents.**—The sera used were pools prepared from 25 to 50 normal human donors. Six separate pools were used, along with a number of individual sera. Aliquots of each were frozen at —70 C and thawed when needed. In some instances the serum was clarified by centrifugation at 35,000 rpm for one hour at 0 C or by filtration through Selas candles of 03 porosity prior to use. In general, R1, R2, and R3 were prepared according to the method of Ecker et al.[13] and were used immediately. R4 was made by incubating 1 cc. of serum with 0.2 ml. of 0.15 N NH_4OH for one hour at 37 C, after which the pH was adjusted to 7.0 with 1N HCl. RP was prepared fresh for each experiment according to the method of Pillemer et al.[14] Hemolytic titrations for the components of complement were performed, in general, by the methods outlined by Pillemer et al.[15]

C. Experimental Technique.—Duplicate flasks were prepared by adding 2 cc. of Medium 29 containing a suspension of 2×10^5 cells to a series

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* The following abbreviations are used throughout the paper:
C', complement;
C'1, first component of complement;
C'2, second component of complement;
C'3, third component of complement;
C'4, fourth component of complement;
RP, serum made deficient in properdin;
R1, serum made deficient in C'1;
R2, serum made deficient in C'2;
R3, serum made deficient in C'3;
R4, serum made deficient in C'4.

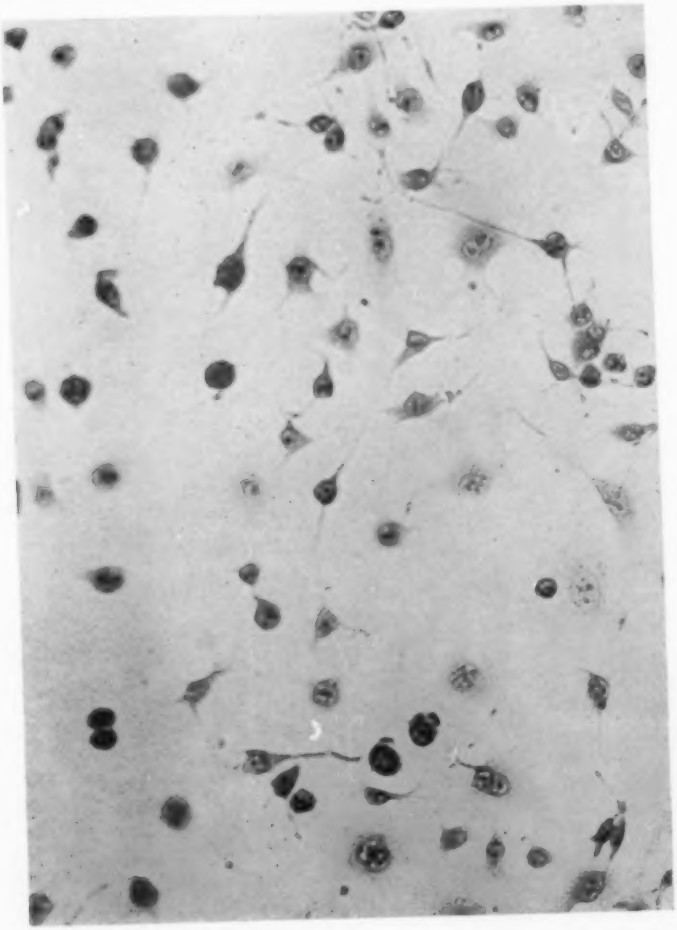

Fig. 1A.—Culture of U12 cells after one-half hour in saline. There are no significant signs of toxicity. Giemsa stain; reduced approximately 20% from mag. ×248.

of T10 flasks.[10] Within one week a film of 5 to 7.5×10^6 cells covered the bottom of each flask. At this time the growth medium was removed from each flask and the cells were washed three times with 5 cc. of warm isotonic saline. The cells were incubated in saline for one-half hour at 37 C and were then examined microscopically to assure that they were in good condition. The saline was removed, and 2 cc. of test reagent was added to the flask. The flasks were stoppered and incubated at 37 C. Microscopic observations for cytologic alterations were made every 15 minutes. The period of observation was usually 3 hours and in some instances as long as 18 hours.

Cytotoxicity was graded as follows: 0 indicates no morphologic difference from saline control (Figs. 1A and 4A); 1+ indicates that the majority of cells displayed retraction of cytoplasmic processes with slight rounding of contour; 2+ indicates that the majority of cells remained intact and displayed wrinkling of the cytoplasmic membrane with slight granularity and vacuolation of the cytoplasm (This was associated with shrinkage of the nucleus and thickening of the nuclear membrane.); 3+ indicates that the cytoplasmic membrane of the remaining cells was poorly distinguished. (There was marked granulation of the cytoplasm. Nuclear pyknosis, karyolysis, and karyorrhexis was frequently observed. An estimated 50% to 75% of the original cell population has disappeared by lysis; 4+ indicates 100% cytolysis. (Few identifiable cells remain on the glass. Foci of granular cell debris are apparent.)

For photomicrography, cultures were fixed in 70% alcohol after removal of reagents and washing in isotonic saline. Some were stained with Giemsa's solution. In other instances, the layer of cells was

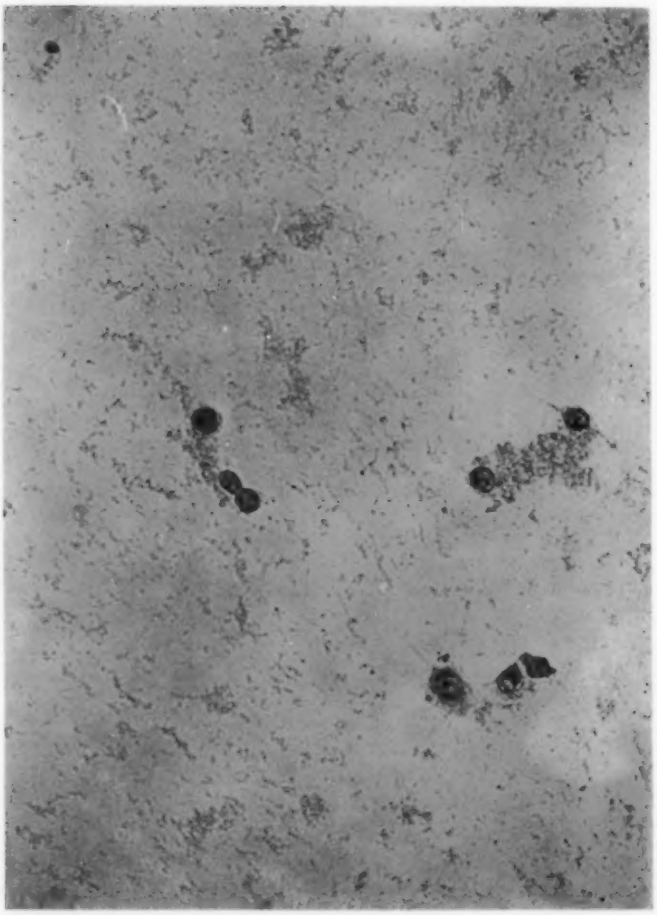

Fig. 1B.—Culture of U12 cells after one-half-hour incubation in undiluted human serum. Note the cytoplasmic granularity of the few remaining cells and the granular cell debris. Giemsa stain; reduced approximately 20% from mag. × 248.

embedded in a thin film of celloidin, which was stained with Giemsa's solution upon removal from the flask. These films were cleared and mounted on slides in the usual manner.

Experiments and Results

General Cytotoxic Phenomena.—1. Cytotoxicity was manifested as a rapid degeneration and necrosis of cells which typically progresses to 100% cytolysis within one hour's incubation. Two plus (2+) toxicity or less was regarded as nontoxic. Intermediary changes were equivocal but rarely encountered. In flasks where 2+ toxicity or less was apparent in one hour, progression of cell degeneration was usually negligible after several additional hours' incubation.

2. All human serum and serum pools tested showed 4+ toxicity within one hour's incubation against the U12-29 cell and HeLa 29 cell, while there was no significant cytotoxicity with the FS strain fibroblast. This toxicity was present in dilutions up to 1:64 in isotonic saline.

3. The U12 and HeLa cells showed no significant toxicity when exposed to undiluted normal rabbit, horse, guinea pig, or rat serum.

4. The cytotoxicity of human serum on the HeLa and U12 cells could be abolished

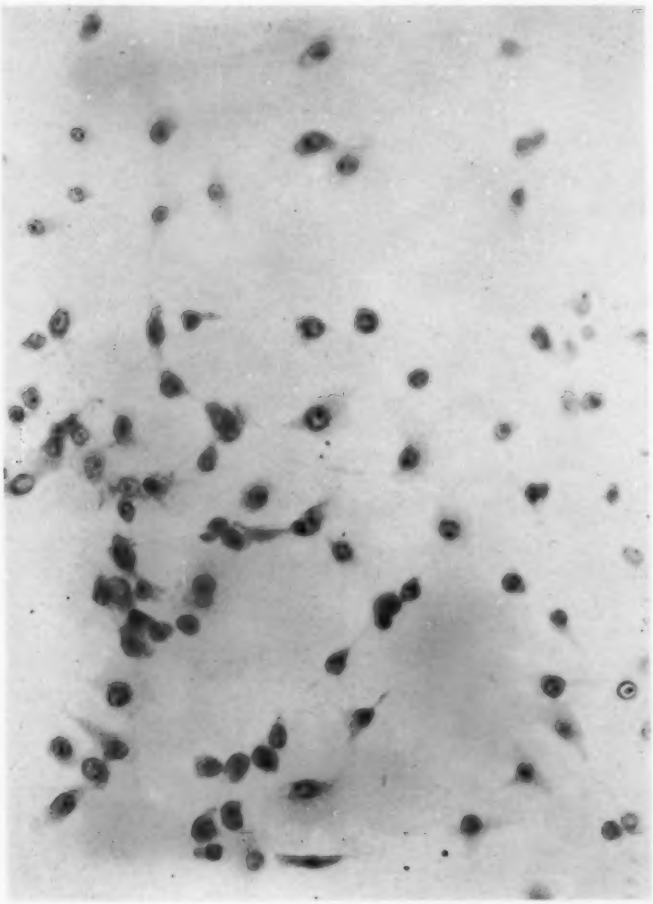

Fig. 2A.—HeLa cell culture after one-half-hour incubation in saline. No significant cytotoxicity is apparent. Hematoxylin and eosin; reduced approximately 20% from mag. × 260.

by heating the serum at 56 C for one-half hour.

5. Dialysis against sodium acetate buffer (μ=0.02, pH 5.5) for 48 hours at 0 C and readjustment of pH and ionic strength (μ=0.15, pH 7.0) failed to alter the toxic effect of normal human serum.

6. The removal of Ca^{++} and Mg^{++} by addition to the serum of edathamil trisodium (Versene) at concentrations of 10^{-3} M or 10^{-2} M failed to influence the toxic reaction.

Properdin-Complement Studies.—All RP reagents tested were as toxic to the HeLa and U12 cells, as was the native serum. Since an RP is known to contain a small amount of properdin (<1 unit per milliliter), it is possible that the observed toxicity reflected an exquisite sensitivity on the part of the cells to tiny amounts of properdin. To test this possibility, it was necessary to prepare a properdin-free serum with intact complement activity. A loss of toxicity from such a serum would strongly implicate properdin in the cytotoxic reaction. Properdin-free serum was prepared with the aid of rabbit antihuman properdin serum.[17] Rabbit antihuman properdin serum † heated to 56 C for 45 minutes to inactivate C′ was absorbed with an equal

† The antiserum was provided by Dr. C. F. Hinz Jr. and Miss Leona Wurz.

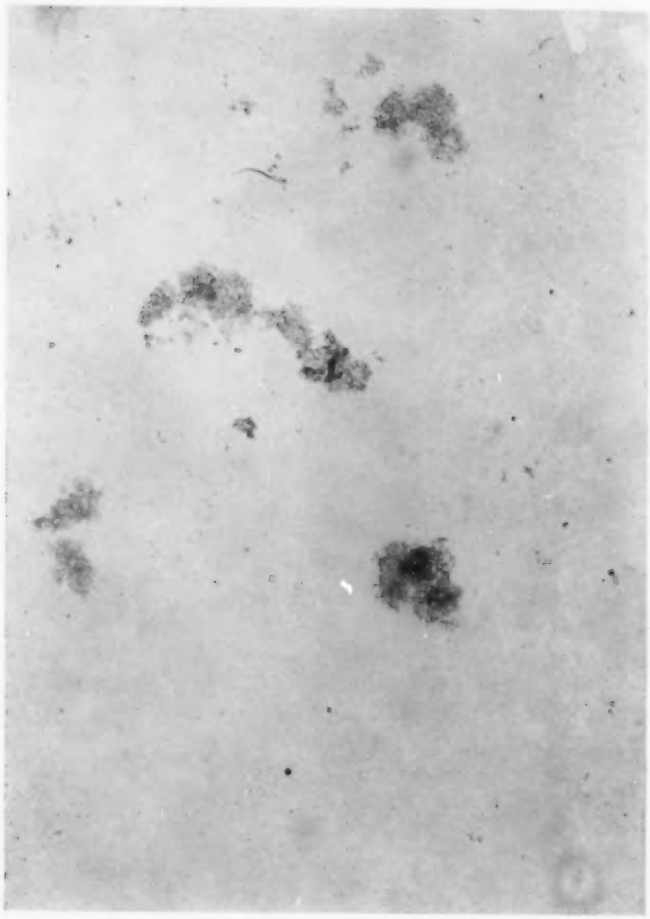

Fig. 2B.—HeLa cell culture after one-half-hour incubation in undiluted human serum. Note the marked granularity, nuclear pyknosis, and debris of cytolysis. Hematoxylin and eosin; reduced approximately 20% from mag. × 260.

volume of human RP at 37 C for one hour and centrifuged at 3000 rpm for one hour at 0 C. One cubic centimeter of the supernatant (adsorbed antiserum) was found to be capable of inactivating 30 to 60 units of human properdin when added to human serum or RP. The adsorption step, designed to remove antibodies not directed toward properdin, involves reactions between antigens and precipitating antibodies, which result in inactivation of the complement originally present in RP (Table 3). For the production of an RP-free of properdin with intact C′, it was necessary to add back increments of RP to the adsorbed antiserum to reestablish C′ activity. The excess of antiproperdin served to combine with the minute amounts of properdin being added back with the RP. Reagents prepared in this fashion demonstrated good complement activity and were found to be as toxic to the HeLa and U12 cells as the original RP (Table 2). On the basis of these observations, it is concluded that the cytotoxicity of normal human serum was probably not dependent on the presence of properdin.

Cytotoxic Activity of Reagents of Complement (R) and Their Combinations.—R1 was as toxic as native serum or RP. Undiluted R2 was toxic, but the toxicity disappeared approximately at a dilution of

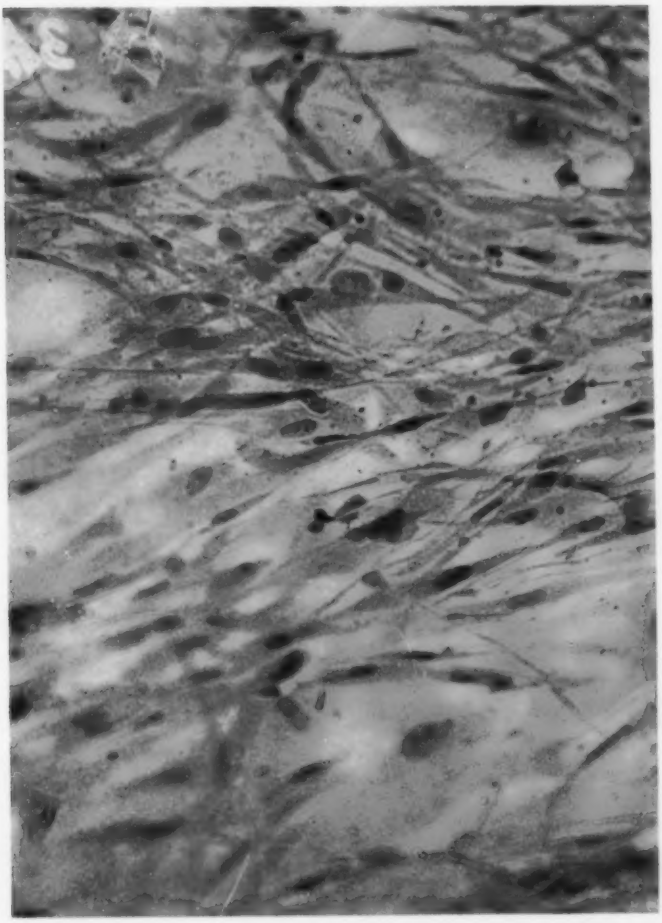

Fig. 3.—FS fibroblast culture after one-hour incubation in undiluted human serum. There is no significant cytotoxicity present. Contrast the morphology of these cells with that of the intact HeLa and U12 cells. Hematoxylin and eosin; reduced approximately 20% from mag. × 260.

1:16. R3 and R4 were essentially nontoxic. Combinations of nontoxic reagents at dilutions of 1:16 reestablished the toxicity, i. e., R3+R4, R2+R4, and R2+R3. These observations are illustrated in Charts 1 and 2.

Effects of Heat Inactivation of Complement.—Heating sera for variable periods of time at 45 C and 56 C resulted in a loss of toxicity on U12 and HeLa cells which coincided roughly with the loss of C'3, C'4, and possibly C'2 activity. The results of these experiments are recorded in Table 2.

Studies with Complement-Fixed Serum. Inactivation of serum complement (C' fixation) was accomplished by exposure of the serum to precipitating antigen-antibody reactions. This was accomplished in two ways:

1. By the addition of 3 cc. of human serum, 0.25 ml. of rabbit antipneumococcal Type III capsular polysaccharide serum and 0.15 ml. pneumococcal capsular polysaccharide Type III solution (1 mg. per milliliter). This mixture was incubated at 37 C for one-half hour and allowed to stand overnight at 0 C. It was centrifuged at 3000 rpm at 0 C for one hour, and the supernatant fluid was removed for testing.

2. By the use of human serum or RP which had been adsorbed with rabbit anti-

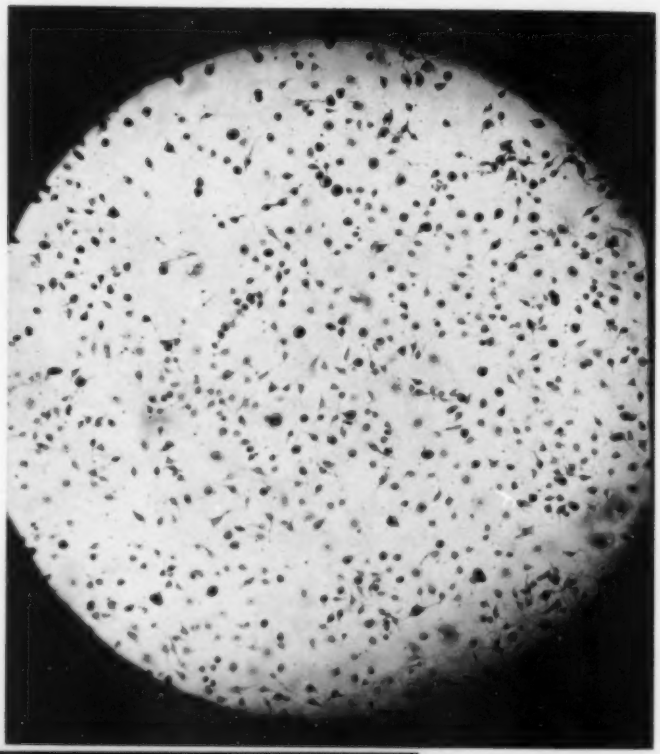

Fig. 4A.—U12 cell culture after one-half-hour incubation in saline. Giemsa stain; reduced approximately 20% from mag. × 64.

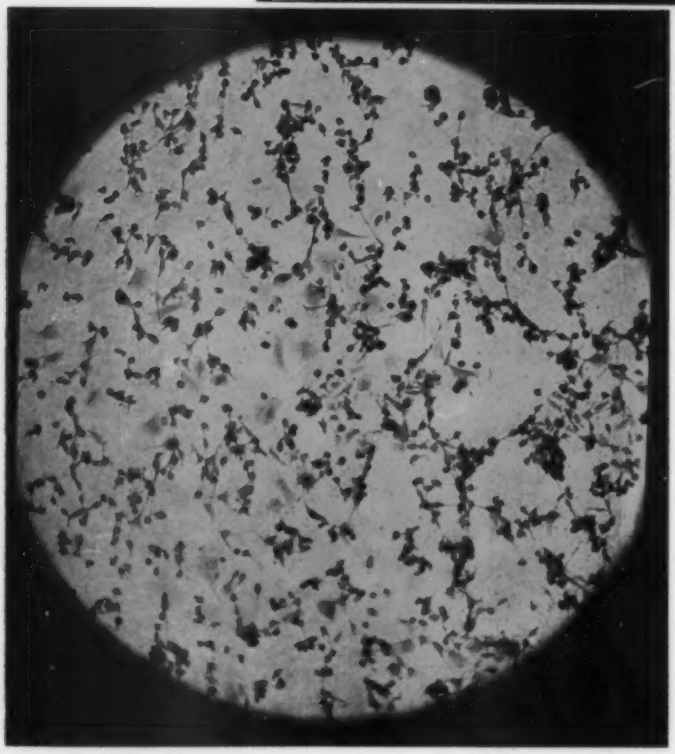

Fig. 4B.—U12 cells after one-half-hour incubation in undiluted guinea pig serum. There is slight clumping and retraction of cytoplasmic processes; 1+toxicity. Giemsa stain; reduced approximately 20% from mag. × 64.

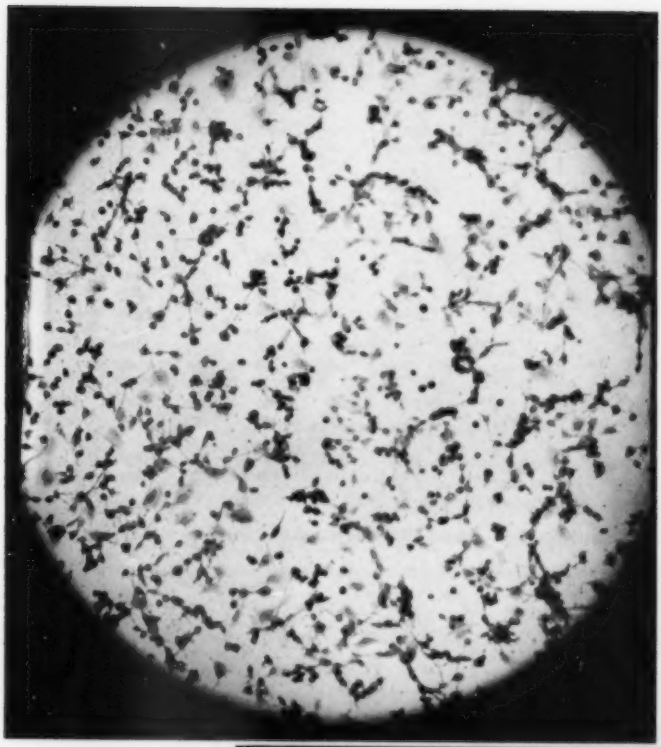

Fig. 4C. — U12 cells after one-half-hour incubation in undiluted human R3. There is 1+toxicity. R4 and heated serum show the same minimal effect. Giemsa stain; reduced approximately 20% from mag. × 64.

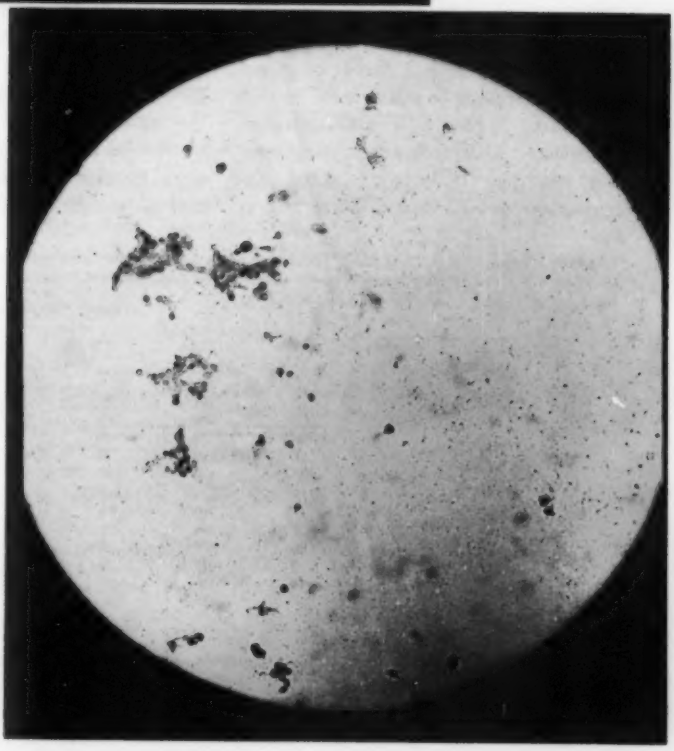

Fig. 4D. — U12 cells after one-half-hour incubation in recombination of R3 and R4 (1:1). There are advanced toxic effects shown by the cells, graded as 3+. Giemsa stain; reduced approximately 20% from mag. × 64.

TABLE 1.—*Exclusion of Properdin by Its Inactivation with Antiproperdin Serum and Reestablishment of C′ Component Activity*

Cell	Reagent, Ml.	AAHP, Ml.*	Saline, Ml.	Final Dilution of Reagent	Titer C′ Components C′1	C′2 Units/Ml.	C′3	C′4	Toxicity in 1 Hr.
U12	0	1	1.5	1:					0
	RP 12								
	1	0	1	1:2	960	120	240	480	4+
	2	1	0	2:3	1,920	240	320	960	4+
	4	1	0	4:5	2,560	480	320	1,280	4+
	8	1	0	8:9	2,560	480	320	1,280	4+
	RP 78								
	1.5	0	1	1:1.5	360	90	120	90	4+
	1.5	1	0	1:1.5 †	240	45	90	60	4+
	Serum 17								
	2	0	0	1:1	2,560	320	320	1,280	4+
	5	1	0	5:6	1,920	160	240	960	4+
HeLa	RP 912								
	3	0	1	3:4	1,920	480	270	840	4+
	3	1	0	3:4	540	120	120	320	4+

* AAHP indicates adsorbed antihuman properdin rabbit serum, 1 cc. of this reagent can inactivate 30 to 60 units of human properdin.

† This mixture was found to be nonbacteriocidal to Salmonella typhi (Felix Type II) organisms as compared to Serum 78 and RP 78, confirming the neutralization of the properdin system.

human properdin serum (see properdin section).

Complement fixation thus performed, resulted in marked reduction in C′2 and C′4 and diminution in C′1 and was generally associated with loss of cytotoxicity. These data are shown in Table 3, and it is apparent that one exception to the general pattern was observed. Serum 12+rabbit antiproperdin remained toxic following the antigen-antibody reaction. It is to be noted that the diminution of C′2 and C′4 are not as marked as in the other situations recorded. The significance of this discrepancy is uncertain.

Comment

Very little work has been done in regard to the nature of serum effects on homologous cells. Carrel and Ebeling [18] noted that the serum of young chickens was superior to that of old chickens for the growth of chick fibroblasts in vitro. Parker [19] showed that a similar effect occurred in human

TABLE 2.—*The Effect of Heat Inactivation of C′ on Cytotoxicity of Serum Pool No. 78*

Cell	Dilution	Temperature	Heating Time, Min.	Titer C′ Components C′1	C′2 Units/Ml.	C′3	C′4	Cytotoxicity in 1 Hr.
U 12	1:1	56C	0	540 *	90	320	1,280	4+
	1:1	56C	5	160	<15	320	1,280	4+
	1:1	56C	15	10	0	120	1,280	4+
	1:1	56C	30	Trace	0	<80	1,280	2+
	1:1	56C	60	0	0	<20	960	1-2+
U 12 & HeLa								
	1:4	45C	0	320 *	60	120	120	4+
	1:4	45C	5	320	60	120	120	4+
	1:4	45C	10	320	60	120	120	4+
	1:4	45C	15	320	30	80	30	4+
	1:4	45C	30	120	<20	60	<20	2+
	1:4	52C	15	60	<20	30	40	2+

* The apparent discrepancy in C′ component titers at 0 time is due to the fact that these experiments were not conducted simultaneously and some variations in component titers are to be expected.

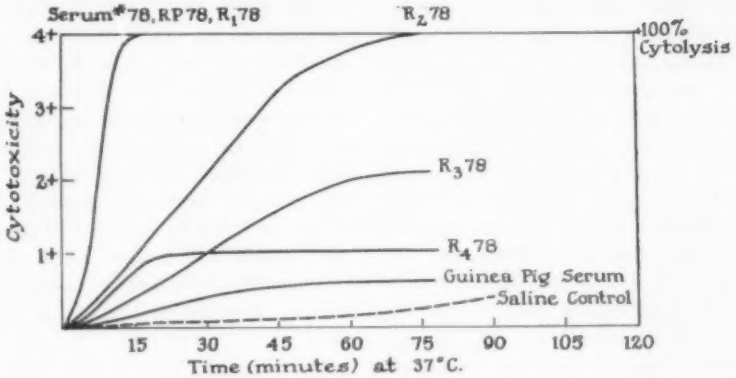

Chart 1.—The cytotoxic reactions of U12 cells to human serum Pool 78 (1:1 dilution) and R1, R2, R3, R4, and RP derived from this pool. Serum and reagents are undiluted. The effect with guinea pig serum is indicated. Saline control is shown as an interrupted line.

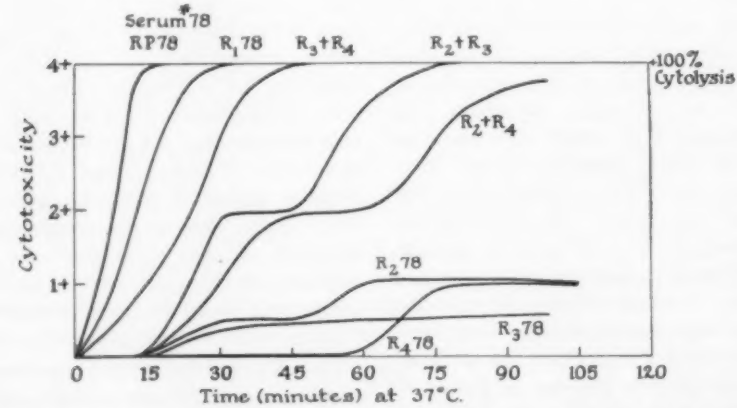

Chart 2.—The cytotoxic reaction of U12 cells to human serum Pool 78 and R1, R2, R3, R4, and RP derived from this pool at 1:16 dilution in saline. The development of toxicity by combinations for nontoxic reagents at this dilution is demonstrated: R3+R4; R2+R3; R2+R4.

TABLE 3.—*The Effect on Serum Cytotoxicity of Inactivation of C' Components by Complement Fixation with Antigen-Antibody Reactions*

Cell	Serum No.	Antigen-Antibody Reaction	Final Serum Dilution	Titer C' Components (Units/Ml.) C'1	C'2	C'3	C'4	Cytotoxicity in 1 Hr.
U12	12	—	1:1	1,200	240	360	720	4+
	12	Pneumococcal Type III polysaccharide + specific rabbit antiserum	1:1	240	<10	240	<10	1-2+
	12	No. 12 + rabbit antiproperdin	3:4	360	20	180	30	4+
	RP 12	—	1:1	1,200	180	240	720	4+
	RP 12	RP 12 + rabbit antiproperdin	3:4	90	<15	90	<15	1+
	RP 66B	—	1:1	1,440	120	120	60	4+
	RP 66B	RP 66B + rabbit antiproperdin	3:4	45	<15	45	<15	1-2+

fibroblasts with infant or adult serum. Chang [20] described a serum factor, toxic to homologous sperm, which he thought resembled complement. Simms and Stillman [21] found that a euglobulin fraction from chicken serum caused degenerative changes in cultures of chick fibroblasts. Fedoroff [22] described a heat-labile factor in the serum of schizophrenic patients which is toxic to mouse fibroblasts (Strain L) but has no effect on HeLa cells growing in a medium containing human serum. Sacerdote de Lustig and Lyonnet [23] and Norris and Majnarich [24] reported that the serum of cancer patients enhanced the growth of human tumor cells in culture, while normal serum had an inhibitory effect.

In the present investigation, normal serum factors appear to destroy atypical cells and cancer cells, while sparing relatively normal cells. It is tempting to speculate that this might reflect the presence of nonspecific factors in serum concerned with natural resistance to cancer. The existence of such factors is suggested by the study of Southam and Pillemer,[3] who found that cancer-cell homografts were rejected by normal volunteers while invasive growth was established in patients with advanced carcinoma. Serious objections must be raised to any pathophysiologic interpretation of in vitro cell studies. The artificial environment of cells growing in culture is physically and chemically very different from that of cells in living tissue. Cells propagated in vitro may undergo extensive metabolic and morphologic alterations in adapting to their artificial environment and typically become very different from their original state in the host. On this basis, it cannot be assumed that cell cultures derived from normal tissue, such as the FS strain, contain normal cells, despite the morphologic similarity of the FS cell to a normal fibroblast in tissue. Nevertheless, such a newly established strain more closely approximates the normal cell in vivo than does the markedly altered U12 cell, which has become more like a tumor cell on prolonged cultivation. The invasive growth potential of the U12 cell has not yet been tested in vivo. It is possible that the cytotoxic action of serum on the U12 and HeLa strains merely demonstrates a sensitivity developed by the cells to human serum while growing on horse serum. Human cells adapted to media containing human serum could not be expected to demonstrate this reaction, and in fact, HeLa cells under such conditions showed no response to normal or schizophrenics' serum in Federoff's study.[22]

The foregoing experiments indicate that the cytotoxic activity of normal human serum on U12 and HeLa cells is not dependent on the presence of properdin but nonetheless appears related to the complement system. R1 and R2 are toxic against these cells, although R2 is toxic to a much smaller extent than R1. The difficulties in preparing an R2 highly deficient in C'2 make interpretation of the role of C'2 from such data tenuous. R3 and R4 are essentially nontoxic. The toxicity of R1 is further evidence against a properdin effect, since R1 is highly deficient in properdin. Furthermore, the lack of dependence of the cytotoxic reaction on Mg^{++} and intact complement would be unlike properdin activity in other systems previously studied.[1]

Considering the results obtained with the reagents of complement, it would seem that the toxicity is related to the presence in serum of C'3, C'4, and possibly C'2. This is borne out to a large extent by the studies with heated and complement-fixed serum. This assertion has only limited significance, however, and must be qualified. The components of complement are defined by their participation in the hemolysis of sensitized red cells. For example, R3 is formed by incubating serum at 37 C with zymosan. During this procedure, numerous serum substances are probably removed or altered. The serum so treated loses both its ability to cause hemolysis of sensitized red cells and its ability to kill U12 and HeLa cells. The substance(s) removed, upon which the

hemolysis of red cells depends, are referred to as C'3. Hence, C'3 activity of a reagent is defined as the relative ability of that reagent to restore hemolytic activity to an R3. The chemical nature of substance(s) removed from serum by zymosan which are responsible for the C'3 hemolytic activity are unknown. An analagous situation holds for the other C' components. In general, the conventional procedures for making R1, R2, R3, and R4 probably alter, in each instance, numerous serum substances, only a portion of which are measured by inhibition of hemolysis. There is no reason to assume that the cytotoxic reaction described is due to the same substances responsible for the hemolysis of sensitized red cells. In this light, the present experiments indicate only that the cytotoxicity depends on the presence of certain factors in normal human serum with properties resembling C'3, C'4, and possibly C'2. Investigations are being undertaken to elucidate some of the problems discussed.

Summary

Normal human serum was observed to be rapidly lethal to two permanent lines of atypical human cells propagated in medium containing horse serum while having no effect on relatively normal short-term cultures of human fibroblasts. Horse, guinea pig, rat, and rabbit sera showed no toxic effect on these cells under the same experimental conditions. The significance of these observations are discussed in relation to natural resistance to neoplastic disease.

The cytotoxicity was shown to be heat-labile and unrelated to the properdin system. The toxic factors resembled C'3, C'4, and possibly C'2 in their behavior toward heat and the various conventional procedures for inactivation of complement.

This work was initiated under the guidance of the late Dr. Louis Pillemer.

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Histochemical Studies on Idiopathic Medionecrosis of the Aorta

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The term "idiopathic medionecrosis of the aorta" has been applied to the histologic picture of a probably degenerative disease which is one of the causes of aneurysm and rupture of the aorta in both young adults and older persons. Gsell [1] first described the condition in 1928, and a little later Erdheim [2] gave it the name. Now the disease is accepted in the textbooks as a distinct pathologic entity.[3,4] The theories concerning its pathogenesis have been reviewed, e. g., by Gore,[5] but the specific cause of the disease is still obscure. The most striking feature is infiltration of the media with a homogeneous mucin-like substance. Authors reporting on the disease speak of "myxomatous," [6] "mucoid," [7] or "hyaline" [4] material after studying it with the classic histologic methods. In the present work, the degenerative changes caused by this disease in the aortic media and especially the homogeneous substance occupying areas of degeneration are studied with the aid of recent histochemical methods and with use of histophysical techniques as controls.

Report of Cases

CASE 1.—A 28-year-old worker died suddenly because of a spontaneously ruptured fusiform aneurysm in the ascending aorta. The clinicopathologic findings of the patient are reported in detail elsewhere.[8] In summary, he had been examined clinically eight years before death. The only pathologic signs revealed were in the cardiovascular system. The clinical diagnosis, aortic valvular insufficiency, was made on the following grounds:

1. Presystolic and diastolic murmurs were auscultated at the aortic area.

2. Increased pulse pressure was present (palpable water-hammer pulse, visible throbbing of the neck vessels, and blood pressure 145 systolic and 70 diastolic).

3. An enlarged heart of aortic configuration was stated.

At autopsy the body was that of a well-developed young man weighing 77 kg. and measuring 184 cm. The hyperemia of the viscera was considered to be a general sign of sudden death. The pericardial cavity was filled with partially clotted blood. The ascending aorta showed a fusiform aneurysm 10 cm. in length involving the aortic ring. This aneurysm was thin-walled and measured maximally 12.5 cm. in circumference. A 1 cm. long transverse break was found 1 cm. above the aortic valves, which were in good condition. The heart weighed 600 gm. This enlargement was almost entirely in the left ventricle. The kidneys weighed 330 gm. There were signs of a slight nephrosclerosis. Other organs showed a normal structure. The clinical signs of aortic valvular insufficiency were considered to be due to the aneurysmal dilatation of the aortic root.

CASE 2.—The patient was a 63-year-old carpenter who had apparently been in good health until he suddenly fell down dead on the floor. There were no clinical observations available. Autopsy showed the body to weigh 71 kg. and measure 170 cm. General signs of sudden death were stated. The pericardial cavity contained approximately 400 ml. of partly clotted blood. The thin-walled ascending aorta was dilated fusiformly and ruptured through a small break within a few millimeters of attachment of the aortic valves. There was no splitting of the media in this case, either. The circumference of the aneurysm measured 10 cm. The heart weighed 370 gm. The kidneys weighed 330 gm. and showed signs of a beginning nephrosclerosis. Except for some pleural adhesions, the other organs were of normal appearance.

CASE 3.—A farmer's wife, 53 years old, who had previously been in good health, was found unconscious on the floor. Twelve hours later she died in a hospital without any accurate diagnosis having been made. At autopsy the body weighed 57 kg. and measured 158 cm. A general hyperemia of the viscera was stated in this case, too. The pericardial cavity contained about 250 gm. of

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clotted blood. The ascending aorta showed a dissecting aneurysm which had finally ruptured into the pericardial cavity through a 5 cm. long transverse fissure 4 cm. above the aortic valves. The heart weighed 350 gm. A slight coronary sclerosis was stated. The kidneys weighed 275 gm. and showed no evidence of nephrosclerosis. There were signs of fibrocaseous tuberculosis in the right lung. The other organs were normal.

In all these three cases serologic tests for syphilis were negative. (Blood samples were taken at autopsy.) Both macroscopic and microscopic examination showed no signs of either atherosclerosis or syphilis in these aortas. No stigmata of Marfan's syndrome were present. The aortic valves were in good condition. No signs of congestive heart failure were stated.

Sections of these aortas were studied histochemically and, in addition, by microincineration and by use of high-resolution microradiography with ultrasoft x-rays.

Methods

The sections were fixed in the buffered 10% neutral formalin of Lillie[9] for 24 hours. The fixed paraffin as usual. Serial sections were cut at about tissue was dehydrated, cleared, and embedded in 10μ and stained with the following methods:

1. Hematoxylin and Van Gieson's stain
2. Weigert's stain for elastic tissue
3. Toluidine blue for metachromasia[10]
4. Lison's Alcian blue for mucopolysaccharides[11]
5. The technique of Rinehart and Abul-Haj for demonstration of acid mucopolysaccharides[12]
6. The original periodic acid-Schiff technique of McManus[13]
7. Hyaluronidase extraction[14] (The commercial preparation Hyalase [Benger] was used. This purified testicular extract is available in ampules each containing 1500 I. U. Prior to use a solution was prepared dissolving the content of each ampule in approximately 3 ml. of 0.85% saline.)
8. von Kossa for "calcium" deposits[15]

In addition, the sections were studied by microincineration for the distribution of the minerals. The technique was described by Hintzsche.[16] Paraffin sections were cut at 5μ, placed on the slides with the aid of liquid paraffin, and incinerated at 520 C for one hour after increasing the heat gradually during three hours. The incinerated preparations were examined by dark-field microscopy, where white granules represent the minerals.

High-resolution microradiography with ultrasoft x-rays was used as a control method to show the distribution of dry weight. Preparations were made according to the technique of Greulich and Engström.[17] Paraffin sections were cut at 2μ. The equipment and the procedure were described by Engström, Lundberg, and Bergendahl.[18] The microradiograms were registered with 1 kv. and 1 ma. The exposure time was 30 minutes. Eastman Kodak Spectroscopic Plates Type 649 were used. Enlargements were made of the developed microradiograms by photomicrography. A relatively high dry weight in a structure is seen as a white area in the microradiogram. Except for minerals, organic substances are represented in the dry weight of a structure.

Results

Histology.—The histologic picture obtained with classic methods (hematoxylin-Van Gieson and Weigert's stain) was similar to previously reported observations.[6,19] In every case noninflammatory lesions were found in the aortic media. Both the elastic laminae and muscle fibers were affected. Elastic tissue had partly lost the staining properties. Some laminae were split or fused together. Especially in the inner and middle thirds of the media the elastic tissue was largely replaced by a basophilic homogeneous substance. Lamina elastica interna was especially destroyed. Cyst formation was not observed. Muscle fibers inserting in elastic laminae were affected, too, and areas showing an absence of muscle cells and nuclei were observed. From the adventitia thin-walled vessels penetrated to the media. The adventitia and the intima were thinner in the walls of the fusiform spontaneously ruptured aneurysms. The intima of the dissecting aneurysm (Case 3) was partly thickened. No signs suggesting syphilis or atherosclerosis were present in the microscopic examination, either.

Histochemistry.—Histochemically, with toluidine blue, the homogeneous basophilic substance infiltrating the degenerated media showed pink (γ) metachromasia. Treated with Lison's Alcian blue technique the same material stained bluish green (Fig. 1). It was found to be bright blue after treatment with the method of Rinehart and Abul-Haj.

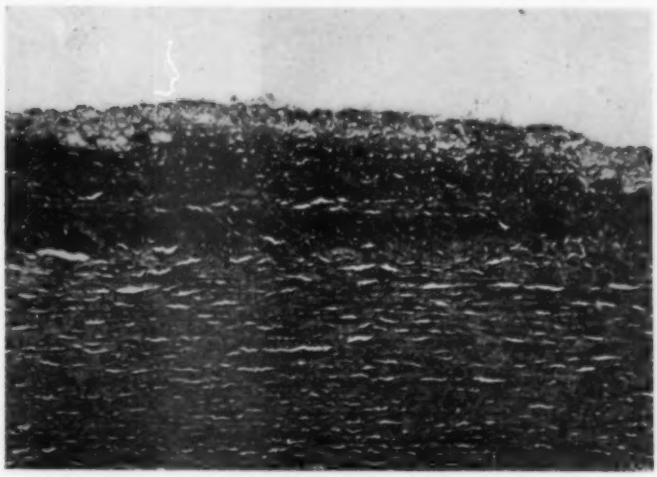

Fig. 1 (Case 1, Idiopathic Medionecrosis).—The homogenous substance in the inner third of the aortic media stains strongly (bluish-green) with Lison's Alcian blue technique. Reduced 15% from mag. × 100.

The substance failed, however, to stain with the periodic acid-Schiff (PAS) technique of McManus. Sections incubated for three hours at 37 C in a solution of Hyalase showed no metachromasia after staining with toluidine blue, but a distinct γ-metachromasia was observed in the control sections incubated at the same temperature for three hours in saline alone and stained analogically.

The von Kossa method for calcium deposits showed no pathologic increased calcification.

Microincineration revealed that the inner part of the media, stated to be infiltrated with basophilic substance, had a relatively small mineral content. In general, the ash content of the aortic media was less than in normal aortas. The ash pattern in medionecrotic aortas (Fig. 2) showed no accumulation of minerals in the region of the lamina elastica interna such as is seen in normal aortas (Fig. 3).

Microradiography showed areas of relatively little dry weight in the inner part

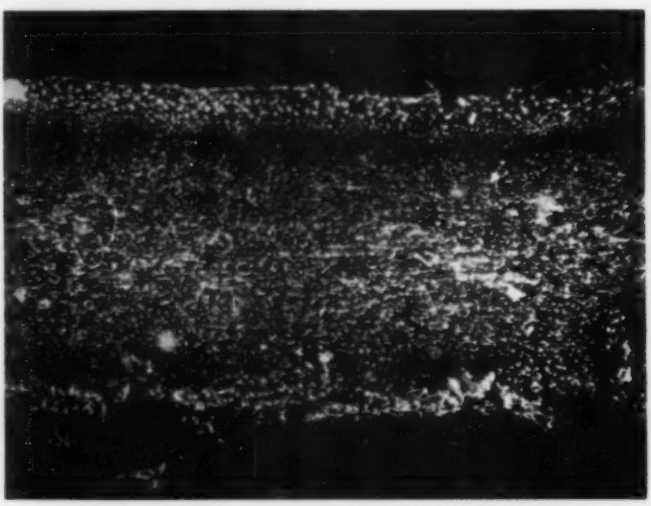

Fig. 2 (Case 1).—Section of the medionecrotic aorta prepared by the microincineration method. The inner part of the media shows a relatively low mineral content. Dark field; reduced 10% from mag. × 58.

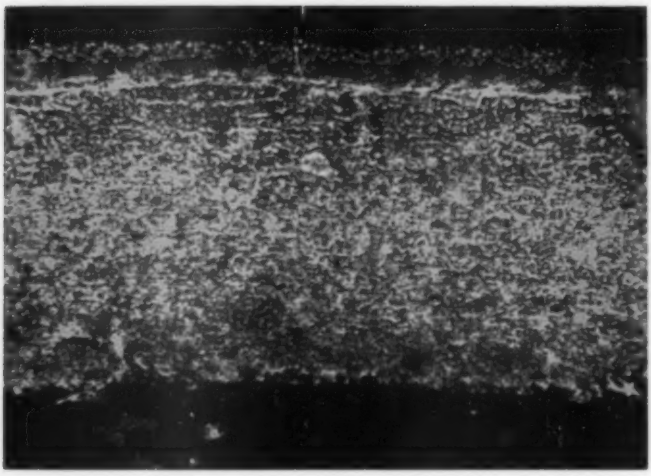

Fig. 3.—Control section of the normal aorta of a 32-year-old man, prepared by the microincineration technique. The white ash represents deposits of minerals accumulated especially in the region of the lamina elastica interna and in other elastic laminae. Dark field; reduced about 15% from mag. × 58.

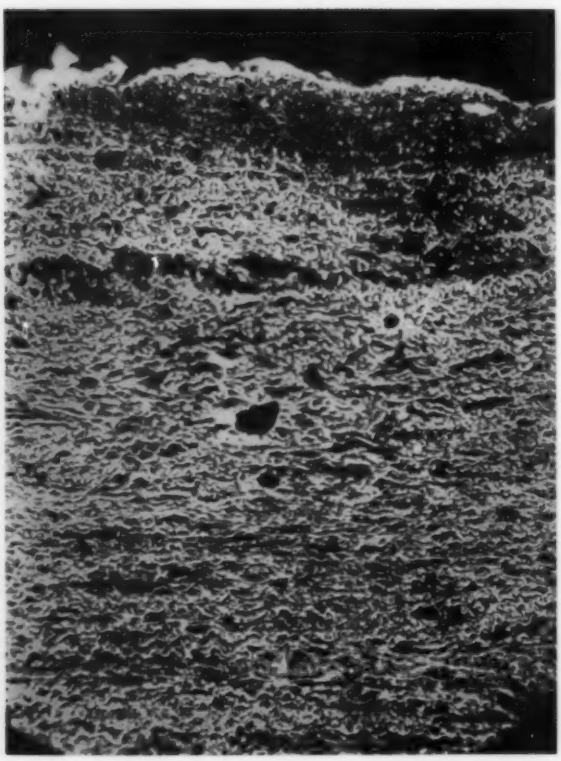

Fig. 4 (Case 1).—Microradiogram of the medionecrotic aorta. Darker areas of relatively small dry weight are seen in the inner part of the media; × 120.

of the degenerated media (Fig. 4). Such areas were not seen in normal aortas.

Comment

Destruction of the elastic tissue in the inner part of the media was stated by histologic methods. The same region had a relatively small mineral content as seen with microincineration. According to Wepler [20] the minerals of the aorta accumulate principally in the elastic laminae. In syphilitic aortitis, in which the elastic elements of the media are destroyed, the amount of calcium is markedly less than in the normal aorta.[21] It seems now that in medionecrosis of the aorta, too, a diminution of minerals occurs simultaneously with the destruction of the elastic tissue in the same part of the aorta.

Areas of relatively little dry weight in the microradiograms correspond roughly with the areas of elastic destruction and of low mineral content.

According to Pearse,[22] the substances which give γ-metachromasia are usually acid mucopolysaccharides. Movat [23] stated that the distribution of Alcian blue corresponds, at least in cardiovascular structures, to the metachromasia seen with toluidine blue. The same was stated concerning their own method for acid mucopolysaccharides by Rinehart and Abul-Haj.[12] Pearse [24] considered that metachromatic material, in fixed tissues embedded in paraffin, whose metachromasia is reversible by one to three hours of treatment with a purified testis hyaluronidase preparation is (1) of a mucopolysaccharide nature and (2) either chondroitin sulfate of Meyer's Types A or C or hyaluronic acid itself or a mixture of any or all of these.

The substance thus consisted of an acid mucopolysaccharide, either chondroitin sulfate or hyaluronic acid. It failed to stain with PAS. The greater part of the material, therefore, ought to be chondroitin sulfate. According to Leblond [25] and Taylor,[26] PAS does not detect this acid mucopolysaccharide on account of substitutions in the glycol groups.

Acid mucopolysaccharides are normally, too, present to some degree in the aortic wall.[27] Kirk and Dyrbye [28] made a biochemical analysis of the total isolated mucopolysaccharide material from human aortic tissue and stated that the greater part of that material consists of chondroitin sulfate.

It seems obvious that in this idiopathic disease the degenerated areas of the aortic media are filled with a substitute, chondroitin sulfate. This acid mucopolysaccharide is to some degree a normal constituent of the aortic wall, but idiopathic medionecrosis causes its augmentation and infiltration. Such an infiltrated aortic wall is not equal to the elastic one. The weakened wall of the ascending aorta cannot withstand the great physiologic pressure acting on this area, and a dissecting or a fusiform aneurysm develops. It may sometimes rupture externally, usually into the pericardial cavity, causing sudden death.

Summary

I have studied histochemically sections of aortas collected from three fatal cases of idiopathic medionecrosis. Microincineration and microradiography techniques were also used. The typical homogeneous substance augmenting in the aortic media was histochemically identified as chondroitin sulfate. Functionally it was considered to be a poor substitute for the elastic tissue. Areas of the worst elastic destruction in the inner part of the aortic media showed a low mineral content and a relatively small dry weight, as seen with microincineration and microradiography methods, respectively.

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The Parathyroid and Experimental Vascular Necrosis in the Rat

1. The Effect of Parathyroidectomy on Experimental Vascular Necrosis

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Reports of numerous experiments indicate the association of experimental renal ischemia, vascular necrosis, and hypertension. It has been demonstrated consistently [1,2] that renal infarction in the rat gives rise to a specific type of vascular necrosis. This necrosis involves principally the small arteries and arterioles and is of the polyarteritis nodosa type. The aorta and large arteries are, however, spared. Hypertension is a frequently associated finding. Lehr and Churg [3] have produced a different type of vascular injury by administering to the rat an excessive amount of a poorly soluble sulfonamide (sodium acetyl sulfathiazole). This agent is believed to act by precipitating in the renal tubules and producing a

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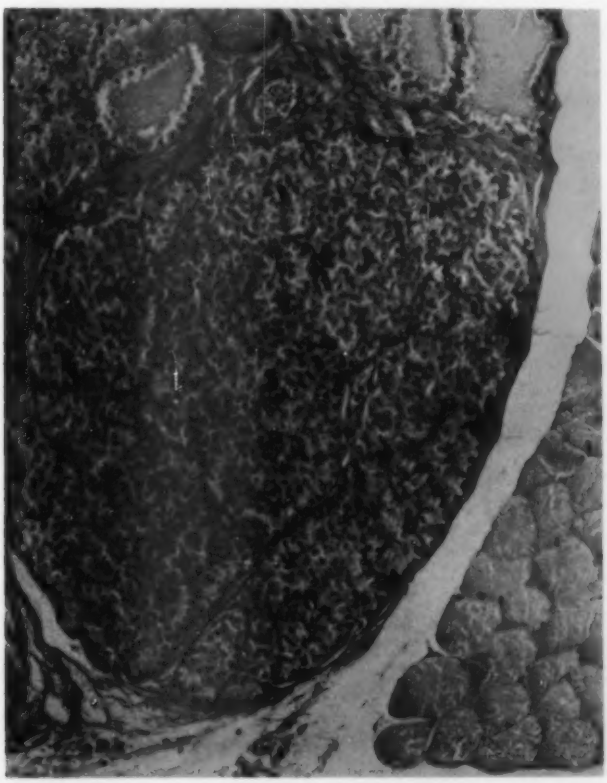

Fig. 1.—Photomicrograph of normal parathyroid gland. Note the thyroid follicles and skeletal muscle bundles at the periphery. Hematoxylin and eosin; × 160.

severe obstructive pyelonephritis. The vascular lesions produced by this type of renal injury are also specific. They consist primarily of degeneration and necrosis of the media of the aorta and large arteries. The lesions appear to be most pronounced in the aortic arch and decrease in severity as the small arteries are approached. The arterioles are spared, and hypertension is transient. Lehr has also demonstrated that this type of renal injury will not give rise to vascular alterations in the absence of the parathyroid glands.[4] He has found that the protection afforded by parathyroidectomy may be overcome by a maintenance dose of parathyroid extract and that excessive doses of parathyroid extract will give rise to the same type of lesions in the intact, nephrectomized, and parathyroidectomized rat.[4]

It is not clear why different types of renal injury appear to give rise to these histologically distinct vascular lesions. The presence of injured renal tissue in both suggested the possibility of a common pathogenesis. An attempt was therefore made to determine whether parathyroidectomy would prevent the particular vascular alterations which appear following renal infarction.

Experimental Procedures

White male and female rats of an inbred Wistar strain, weighing 150-300 gm. and 2-3 months of age, were utilized. Standard renal injury, a modification of Koletsky's technique,[3] was produced in the following way. A figure-eight silk ligature was applied to the left kidney. The ligature was tight enough to insure focal parenchymal necrosis in almost every animal. The right renal pedicle was completely ligated, with no attempt to isolate the

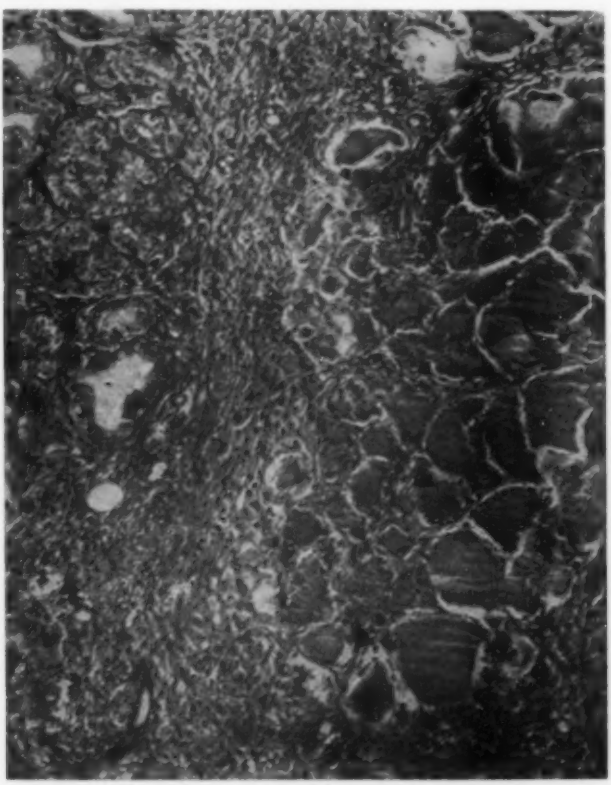

Fig. 2.—Histologic section from site of parathyroid cauterization after nine days. Collections of thyroid colloid are surrounded by leukocytes, phagocytes, and fibrous tissue. Intact thyroid follicles are visible at one side. No parathyroid tissue is visible. Hematoxylin and eosin; × 125.

Fig. 3. — Normal aorta from rat killed six days after standard renal injury. Hematoxylin and eosin; × 160.

renal artery or vein. Parathyroidectomy was performed by cauterizing both readily visible parathyroid glands. A narrow border of surrounding thyroid tissue was included to insure complete destruction.

The control rats, numbering 37, were subjected only to the standard renal injury. The experimental animals, numbering 40, were subjected to parathyroidectomy in addition to the standard renal injury. Parathyroid cauterization was performed two days prior to renal injury in 3 rats, four days prior to renal injury in 14 rats, nine days prior to renal injury in 12 rats, and at the same time as the renal injury in 11 rats.

Blood pressures were determined in 20 control and 37 experimental animals, with use of the tail plethysmograph. All had preoperative base-line blood pressures taken for three consecutive days. Readings were begun again, after renal injury, on the third postoperative day and were repeated every other day until the time of killing.

Surgical procedures were performed with use of ether anesthesia and clean technique.

All surviving animals were killed, with use of light ether anesthesia, 6-15 days after renal injury, by decapitation or rapid exsanguination by cannulating the abdominal aorta. Histologic sections were prepared from heart, aorta, kidneys, pancreas, mesentery, adrenals, and skeletal muscle. The neck organs of seven of the experimental rats were imbedded *in toto* and serially sectioned to determine whether all parathyroid tissue had been destroyed. Tissues were fixed in Zenker's formalin or 10% formalin, and sections were stained with hematoxylin and eosin.

Serum calcium determinations, with use of the potassium permanganate titration technique of Clark,[6] were performed on blood collected from most of the surviving rats at the time of autopsy.

Diet consisted of GLF Big Red Dog Pellets and tap water, both provided in liberal quantities for ad libitum consumption. No attempt was made to supplement the diet of the parathyroidectomized rats with additional calcium. The animals were housed in separate wire-mesh cages during the experimental period.

Results

The mortality rate was exceedingly high among both the control and the experimen-

tal animals. Of the 37 control rats (subjected only to the standard renal injury), 20, or 54%, survived until the time of killing. Of the 40 experimental animals (subjected to parathyroid cauterization and standard renal injury), only 12, or 30%, survived until the time of killing. Of the animals that died before the time of killing, all but one died between 24 and 48 hours after the renal injury. Autopsy consistently revealed considerable serosanguineous peritoneal and pleural effusion, as well as pulmonary congestion, edema, and hemorrhage. The mortality rate among the experimental animals was not appreciably affected by changing the time between parathyroid cauterization and renal injury.

Examination of the serially sectioned neck organs of seven of the experimental rats revealed no identifiable parathyroid tissue. The sites of cauterization revealed pronounced architectural and cytological alterations of thyroid follicles and surrounding connective tissue elements (Figs. 1 and 2). Although severe, the histological alterations were well localized, and the greater portion of the thyroid gland was normal.

In all rats, the right kidney, the pedicle of which had been ligated, was moderately enlarged and completely infarcted. The figure-eight ligature produced focal lesions in the left kidney, which varied from cortical atrophy to frank necrosis. The extent of these lesions varied somewhat from animal to animal, but some injury was always present.

Careful examination of the gross organs at autopsy revealed no visible alterations. Microscopic vascular necrosis of the polyarteritis type was present in all of the control rats and in all but two of the ex-

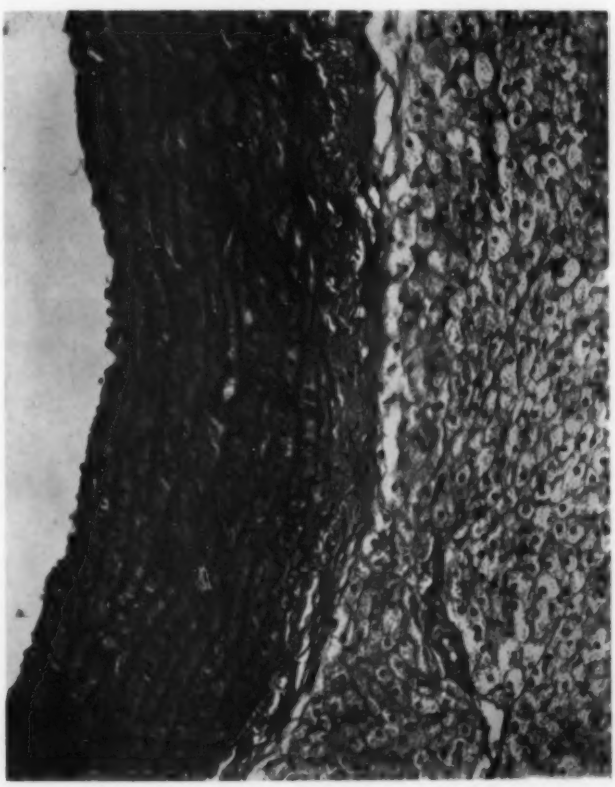

Fig. 4. — Normal aorta from rat with parathyroid cauterization killed eight days after standard renal injury. Hematoxylin and eosin; × 160.

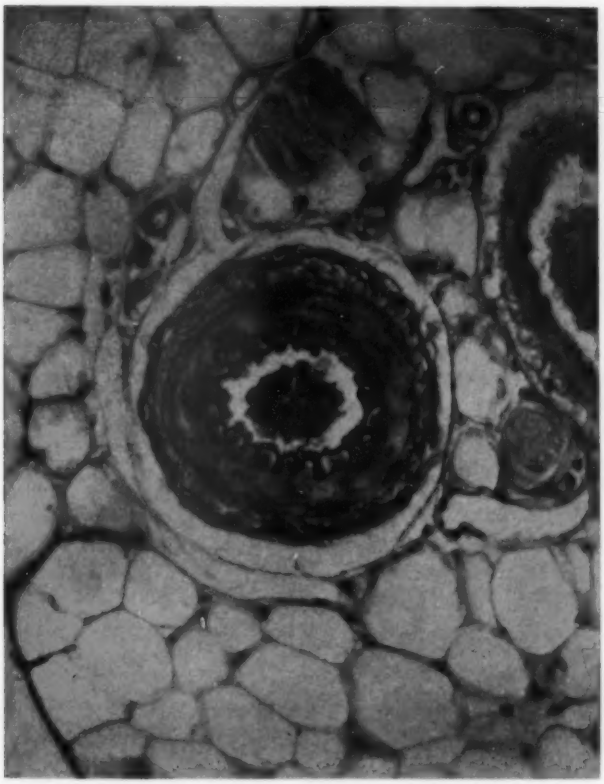

Fig. 5.—Photomicrograph of a normal mesenteric artery. Note the distinct internal elastic membrane and the smooth muscle fibers of the media. Hematoxylin and eosin; × 336.

perimental animals. There appeared to be no significant reduction in the number of lesions present in the animals which had undergone parathyroid cauterization, with the exception of the two without lesions. The animals in which the figure-eight ligature produced the severest renal injury usually revealed the most numerous and most striking vascular necrosis. In the two rats without vascular necrosis, the left kidney revealed only minimal evidence of damage.

The lesions, when present, were always to be found in either the vessels of the pancreas or mesentery and usually in both. Occasionally, periadrenal vessels were involved. Less frequently, foci of myocardial degeneration and necrosis were visible. An associated small artery or arteriole showing necrosis was not always visible. Only a rare section of skeletal muscle revealed the vascular alterations. No vascular lesions were present in the sections of neck organs or aorta (Figs. 3 and 4).

The necrosis appeared as a ring of brightly eosinophilic homogeneous material replacing principally the innermost portion of the media. The endothelium was usually intact. The extent of the necrosis was variable from vessel to vessel and often replaced the entire thickness of the wall. The wall and its periphery were infiltrated by polymorphonuclear leukocytes, lymphocytes, eosinophils, and monocytes, together with fibroblasts and collagen (Figs. 5, 6, and 7). With time, the peripheral fibrosis increased in extent, giving the lesion the characteristic nodular appearance.

The blood pressure of the normal Wistar rats in our colony varies within a range of

90-110 mm. of mercury, with an occasional reading of 80, 120, and as high as 130. Prior to renal injury, the blood pressures of all of the control and experimental animals were within the range cited. Parathyroid cauterization did not affect the blood pressure. After renal injury, the blood pressure of only three of the surviving control rats reached 140 mm. of mercury. That of the remaining control rats and of the experimental animals stayed within the preoperative normal range (Table 1).

Serum calcium determinations were performed on 10 control and 10 experimental animals. The average value for the control rats was 12.4±1.5 mg. %, while that of the experimental rats was 9.4±1.2 mg. % (Table 2).

Comment

It is evident from the very high mortality rate and the high incidence of vascular necrosis that the degree of renal damage inflicted by the experimental procedure was severe. This particular method was elected to insure the appearance of vascular alterations in all of the control animals and hence provide a clear-cut base line upon which an accurate appraisal of the effect of parathyroid cauterization might be possible. The pulmonary congestion, edema, and hemorrhage, together with the serosanguineous pleural and peritoneal effusions found in all animals dying between 24 and 48 hours after renal injury, might be considered to indicate an overwhelming acute renal failure as the cause of death.

The results clearly indicate the failure of parathyroid cauterization to prevent the appearance of necrotizing vascular disease following renal infarction in the rat. This indicates that parathyroid secretion plays

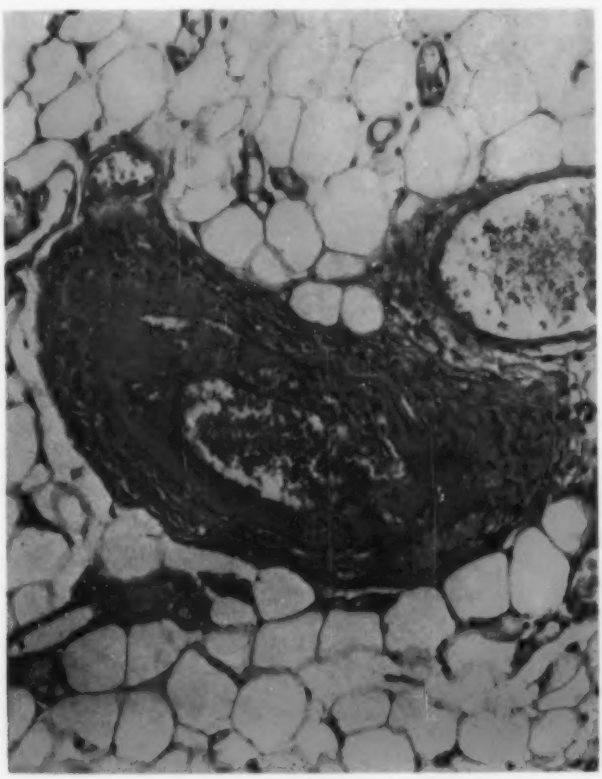

Fig. 6.—Necrotic mesenteric artery from rat killed seven days after standard renal injury. Hematoxylin and eosin; ×160.

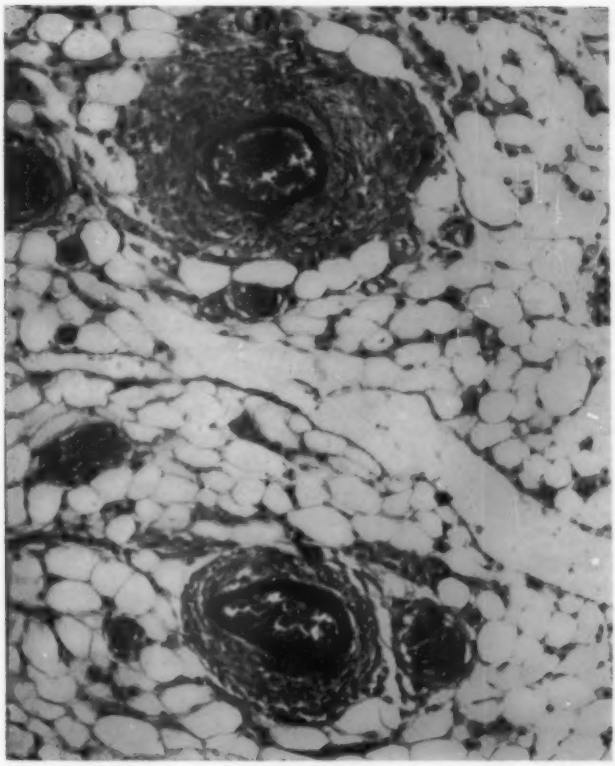

Fig. 7.—Necrotic mesenteric arteries from a rat with parathyroid cauterization killed seven days after standard renal injury. Hematoxylin and eosin; × 160.

no part in the initiation of the necrotizing vascular disease associated with renal infarction. This further indicates that the vascular disease produced by renal infarction is pathogenetically different from that produced by sulfonamide-induced "obstructive nephropathy." The results of this experiment, however, do not help to explain why the kidney, when injured by these different techniques, appears to initiate two distinct types of vascular disease.

The absence of hypertension in all of the control and experimental animals is worthy of comment. By substituting ligation of the renal pedicle for the selective renal artery ligation of Koletsky's technique, hypertension has not occurred. It can therefore be proposed that the ligation both of the renal artery and of the vein serves to partially exclude from the blood stream a substance, produced by the infarcted renal parenchyma, which has been implicated in the etiology of hypertension and necrotizing vascular disease. This theory was advanced by Goldblatt, who prevented hypertension by clamping both the renal vein and the renal artery in the dog.[5] If this theory were applied to the present experiment, it would be necessary to assume that the quantity of substance escaping was sufficient to produce vascular necrosis but insufficient to produce hypertension. On the other hand, it can be proposed that the absence of hypertension in these animals with vascular necrosis demonstrates that the two phenomena are not dependent upon each other.

The choice of cauterization as a method of destroying the parathyroid tissue was made because of the ease with which it can be performed, the minimal damage to thyroid tissue, and the absence of bleeding

TABLE 1.—*Blood Pressure Response in Control and Experimental Animals*

Rat	Procedure	Preoperative Blood Pressure	Blood Pressure at Killing
1	Standard renal injury	100	120-130
2		90-100	130-140
3		120	130
4		110-120	140
5		110	120
6		110	130
7		110-120	130-140
8		100	110
9		110	130
10		110	130
11	Parathyroidectomy & standard renal injury	100	130
12		100	110
13		100	120-130
14		90	100-110
15		90-110	100-110
16		90-100	120-130
17		110-130	100-110
18		100	130
19		90-110	130

TABLE 2.—*Serum Calcium in Control and Experimental Animals*

Rat	Procedure	Serum Calcium, Mg. %	Average, Mg. %
1	Standard renal injury	--	12.4±1.5
2		11.9	
3		13.6	
4		13.3	
5		13.9	
6		11.8	
7		11.9	
8		11.4	
9		11.5	
10		12.1	
11	Parathyroidectomy & standard renal injury	8.5	9.4±1.2
12		8.1	
13		10.4	
14		8.7	
15		8.7	
16		10.4	
17		8.8	
18		9.9	
19		10.6	

at the time of operation. The moderately reduced serum calcium levels in the parathyroidectomized rats are in conformity with the work of Shelling[7] and Tweedy,[8] who found that the serum calcium is considerably lowered during an initial period of several days but that subsequently the rat will maintain low normal levels, unless starved or given a diet deficient in calcium.

Summary

Necrotizing vascular disease has been produced in the rat by unilateral renal infarction and contralateral figure-eight ligature. Cauterization of the parathyroid glands prior to renal injury did not prevent the appearance of the vascular necrosis. These results suggest that the pathogenesis of the lesions produced by renal infarction differ from those produced by sulfathiazole-obstructive pyelonephritis, which are prevented by parathyroidectomy.

The absence of hypertension in rats with arterial necrosis is discussed.

Miss Harriet L. Feary provided technical assistance in the preparation of the microscopic sections.

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Effect of Radiothyroidectomy in the Parakeet

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Tumors of the pituitary gland are among the commonest neoplasms of the shell parakeet, Melopsittacus undulatus.[1] During the past four years over 180 cases of pituitary adenoma or carcinoma have been examined in this laboratory. The etiology and pathogenesis of the tumors remains obscure. The development of pituitary tumors in C57BL mice following radiothyroidectomy is well known,[2,3] but this method is not equally successful in other strains of mice and in rats,[2] nor has it produced pituitary tumors in dogs.[4] Among birds, radiothyroidectomy has been employed in fowl chicks[5] and the Oregon junco[6] but no note made of changes in the pituitary. In view of the frequent occurrence of pituitary tumors in the parakeet, a study of the effect of radiothyroidectomy on this bird was undertaken.

Materials and Methods

Seventy-two parakeets of both sexes, 3-4 months old, each weighing about 25 gm., received an intramuscular injection of 300μc. of I^{131} in 0.2 ml. of saline. The birds were fed equal parts of canary and millet seed and water ad libitum. Hulled oats coated with cod liver oil were given once a week; carrot greens, on alternate days. Metabolic rates were determined with a closed-circuit apparatus similar to that described by Benedict[7]; it was originally used by Dr. Robert Grubbs, Department of Physiology, The Ohio State University, to determine the basal metabolism of rats. His advice and help were given while these studies on the parakeet were carried out. Each bird was placed in a pint Mason jar closed with a brass lid and rubber gasket. Two holes in the lid were fitted for the attachment of rubber tubing to permit gas exchange. The jars were placed in a constant-temperature water tank at 22 C. Air was circulated through the jars and the CO_2 absorbant chambers by oil-immersed pumps; Baralyme was the CO_2 absorbant. Oxygen was supplied from a bank of eight spirometers, each consisting of a brass tube 3.81 cm. wide and 20.1 cm. long and a brass bell suspended over a pulley by a thread. A pointer attached to the counterweight passed in front of a vertical millimeter scale; 1 mm. on the scale represented 1.16 cc. of oxygen. Four spirometers, each in turn connected to the circuit for five minutes, were used for each determination. The respiratory quotient used was 0.73. After corrections were made for barometric and vapor pressures the results were expressed in calories per gram per day using the formula:

$$\text{scale reading} \times CF(11.6) \times \frac{P}{T} \times CaF(0.73) \div \text{weight (gm.)},$$

where CF is the calibration factor of the spirometer (11.6), P is the barometric pressure minus vapor pressure at the average temperature T of the spirometer, and CaF is the caloric factor (0.73).

After death the birds were examined promptly and the tissues fixed in 10% formalin. After fixation the pituitary and adrenals were weighed on a Roller-Smith torsion balance. Histological sections were routinely prepared of the pituitary, adrenals, pancreas, heart, lungs, liver, spleen, kidneys, gonads, and femur.

Results

Thyroid.—In the parakeet the thyroid is a paired organ, one gland lies on each side of the neck near the base, between the common carotid artery and the jugular vein. The normal size is 2-3 mm.×1-2 mm. Two parathyroids, often confluent, appear as minute orange nodules at the lower pole of each thyroid. Within five days after injection of I^{131} the thyroids are grossly hemorrhagic; microscopically the entire gland is necrotic. The parathyroids occasionally show evidence of cell damage in the areas immediately adjacent to the thyroid, but after two to three weeks they again appear normal. Within a month the

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Fig. 1. — Fibrosis and hyalinization of thyroid site 26 days after intramuscular injection of 300μc. of I^{131}. The wall of the internal carotid artery shows slight fibrosis and scant lymphocytic infiltrate; the lumen is almost filled with normal nucleated erythrocytes. Hematoxylin and eosin; reduced about 25% from mag. × 80.

necrotic thyroid is wholly resorbed and replaced by fibrous tissue (Fig. 1). A few chronic inflammatory cells are present, and the walls of small vessels in this region are the seat of hyaline thickening. Destruction of the thyroid is complete, and histological examination of blocks of tissue that included the parathyroid for identification never contained a trace of thyroid tissue.

Metabolism.—After destruction of the thyroid the birds were less active than usual and sat quietly on their perches, often with slightly ruffled feathers, as normal birds do when cold. The cloacal temperature at a depth of 1 cm. was measured with an electric thermocouple. In seven male and five female euthyroid parakeets the temperature range was 107-109.5 F, with an average of 108.0 F. The temperatures were recorded at 9 a. m. and 4 p. m. for a period of 14 days. Similar measurements made on two male and two female birds 18 months after injection of I^{131} gave a range of 101.0-106.5 F, with an average of 103.7 F. These results indicate that the body temperature of thyroidectomized parakeets averages more than 4 degrees (F) below that of the normal birds. The much wider range of temperatures recorded would also indicate an impairment of heat control. In this respect it may be noted that very young parakeets,[8] like other birds hatched in a very immature condition,[9] are poikilothermic; homothermy is not established until the nestlings are two weeks old. In adult pigeons subjected to surgical thyroidectomy Marvin and Smith observed a drop in basal heat production within four to five days after removal of one thyroid; further depression followed excision of the second gland.[10]

Although very many determinations of the metabolic rate were carried out on normal and thyroidectomized parakeets, probably none of the results obtained were basal. The tests were made in the forenoon in a darkened room to which the birds had been transferred on the previous afternoon. For 15 hours before the test the birds were given water but no feed. During the test the tank was covered to exclude light. The results obtained appear high when compared with basal metabolic rates of other animals and birds.[7,11] Nevertheless, a definite drop in heat production was clearly evident following injection of I^{131}, whereas no consistent change was found in controls repeatedly tested over a period of several months. Table 1 lists the results of a series

TABLE 1.—*Heat Production Before and After Radiothyroidectomy* *

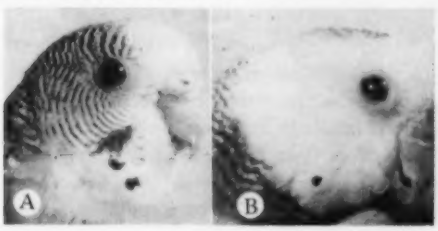
Bird No.	Date									
	6/15	6/16 †	6/23	6/30	7/7	7/14	7/28	8/11	9/8	10/6
1	0.484	I^{131}	0.437	0.313	0.316	0.278	0.301	0.307	0.312	0.244
2	0.698	I^{131}	0.501	0.410	0.439	0.388	0.358	0.362	0.317	0.320
3	0.690	I^{131}	0.581	Died						
4	0.617	I^{131}	0.543	0.183	0.324	0.319	0.319	0.354	0.369	0.266
5	0.608	I^{131}	0.562	0.336	Died					
6	0.519	I^{131}	0.412	0.431	0.380	0.344	0.344	0.352	0.291	0.178
C-1	0.544	Control	0.572	0.587	0.505	0.502	0.579	0.534	0.532	0.546
C-2	0.480	Control	0.453	0.430	0.540	0.541	0.503	0.496	0.547	0.541
C-3	0.523	Control	0.557	0.433	0.439	0.548	0.442	0.463	0.427	—
C-4	0.510	Control	0.476	0.611	0.456	0.581	0.542	0.531	0.549	—

* Values given as calories per gram in 24 hours.
† Date on which I^{131} was injected.

of tests in a representative group of treated and control birds.

Plumage.—Two months after administration of I^{131} color changes became apparent. These were most striking in the head and breast feathers, where blue and black, due to melanin, faded or wholly disappeared (Fig. 2). The role of the thyroid in feather pigmentation has been exhaustively studied in the fowl, particularly the Brown Leghorn.[12] In the fowl there is pronounced sexual dimorphism, but in parakeets no difference in plumage is noted between the male and female. The melanin pigments are synthesized by the melanoblasts and transferred to the epidermal barbule cells of the feather germ. In the Brown Leghorn male thyroidectomy leads to the disappearance of black melanocytes and their replacement by cells with yellow or orange granules; this process can be reversed by administration of thyroxin. The mechanism of color change in the parakeet has not been investigated.

Fig. 2.—*A*, appearance of normal hen before administration of I^{131}. *B*, same bird as in *A*, nine months after injection of I^{131}. Note loss of pigment and slight periorbital edema.



Structural changes were also observed in the feathers of thyroidectomized parakeets. They consisted of a striking elongation and narrowing of the tail feathers; similar changes in the small feathers of the back and wings gave them a hair-like appearance (Fig. 3). Regrowth of feathers is defective, and moulting is delayed; the bird may become partly bald and has a generally unkempt appearance. These effects of thyroidectomy have been thoroughly investigated in the chicken,[13] where the gross changes observed in the feathers are due to failure of barbule development. The same relationship between thyroid and plumage has been observed in pigeons[14] and ducks.[15]

Skin changes that resemble those of human myxedema are not seen in the athyroid parakeet, although they are found in radiothyroidectomized dogs.[4,16] The epidermal atrophy characteristic of myxedema in the dog would be difficult to recognize in the parakeet, for in the latter the normal epidermis is only one to three cell layers thick. In some instances slight hyperkeratosis and periorbital edema (Fig. 2*B*) were noted. The dermis was of normal thickness, and the periodic acid-Schiff (PAS) stain failed to show an increase in mucopolysaccharides.

Pituitary.—In the radiothyroidectomized parakeets the weight of the pituitary in-

Fig. 3.—Plumage changes five months after treatment with I^{131}. The hair-like character of some feathers is seen in the bird in foreground. The one behind it shows the ruffling of feathers often displayed by the athyroid parakeet.

creased with the length of survival. Pituitary weights were obtained in 53 treated and 54 control birds that survived for periods of 70 to 800 days. In the former the mean weight of the pituitary from 70 to 400 days after injection of I^{131} was 1.06 mg.; for the next 400-day period it was 1.29. In the control birds the weights were 0.92 mg. and 0.72 mg., respectively (Table 2). By the *t*-test of Snedecor [17] the difference in the pituitary weights of radiothyroidectomized and control birds during the 401- 800-day interval was highly significant, with a *P* value of less than 0.001.

TABLE 2.—*Pituitary Weights of Radiothyroidectomized Birds (I^{131}) and Controls*

	Birds, No.	Survival Period, Days	Pituitary Weight, Mg.	*P* Value *
I^{131}	17	70-200	1.03	
Control	17	70-200	0.76	<0.05
I^{131}	18 †	201-400	1.06	
Control	22	201-400	0.92	>0.05
I^{131}	18 ‡	401-800	1.29	
Control	15	401-800	0.72	<0.001

* Probability by *t*-test of Snedecor.
† Two cases of pituitary tumor not included.
‡ One case of pituitary tumor not included.

The cell composition of the anterior pituitary in the parakeet is similar to that of other birds [2-8] and has the usual complement of acidophils, basophils, and chromophobes. The acidophils in bird pituitaries are of two types, namely, darkly staining A_1 cells and lightly staining A_2 cells. The latter may be difficult to distinguish from chromophobes. Within three months of radiothyroidectomy, cells resembling the A_2 acidophil have increased in number. They are larger than the A_2 cells of the normal pituitary, with a more abundant cytoplasm. When fixed in Bouin's fluid and stained with Heidenhain's azan (azocarmine G), the cytoplasm is seen to contain faint metachromatic granules (Fig. 4).

Similar "thyroidectomy" cells have been described in the pituitaries of dogs [4] and mice; in the latter they have been identified as amphophils.[19] In a long series of studies on the fowl pituitary Payne first regarded the thyroidectomy cells as modified chromophobes,[20] but in a later publication he

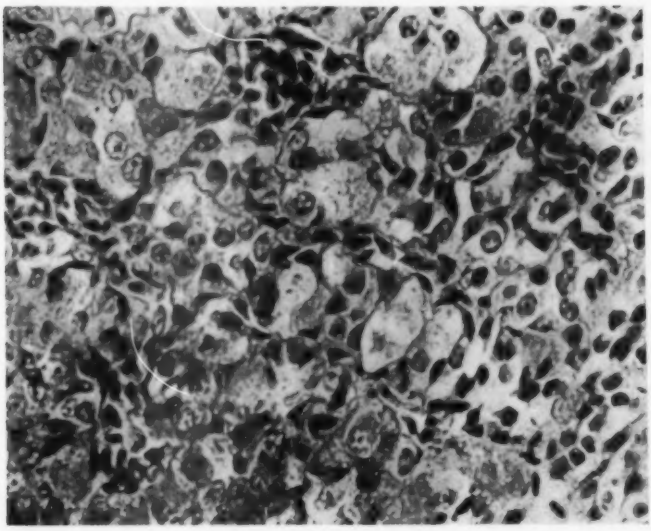


Fig. 4.—Section of pituitary that weighed 1.15 mg.; bird received I^{131} 54 weeks earlier. Large pale cells with faint granules are "thyroidectomy" cells, small oval darkly nucleated cells are erythrocytes. Heidenhain's azan stain; reduced about 10% from mag. × 900.

pointed out that their nuclear structure and site of origin are similar to that of acidophils.[21] The derivation of these cells in the parakeet is still in doubt; tentatively they may be regarded as variants of A_2 acidophils.

No pituitary tumors were found in the 128 control parakeets at any time; 81 of these survived over 200 days. Among the 72 I^{131}-treated birds, 37 lived over 200 days; in 3 of these pituitary tumors were found at autopsy. The first tumor occurred in a male bird that died 305 days after radiothyroidectomy. It measured 4×5 mm. and had invaded the bone of the sella. The second case was a female that died 343 days after I^{131} injection. The pituitary was four times normal size and had been diffusely replaced by a tumor that showed early invasion of the cancellans bone of the sella. The third tumor was found in a parakeet that died 636 days after radiothyroidectomy. The pituitary was × 2 normal size, and histological sections showed that two-thirds of the gland had been replaced by an adenoma (Fig. 5). In the kidney was

Fig. 5. — Adenoma in pituitary of bird 23 months after injection of I^{131}. Hematoxylin and eosin; reduced about 10% from mag. × 40.

a 2×2 cm. poorly differentiated adenocarcinoma.

Histologically the pituitary tumors resembled those observed to occur spontaneously in the parakeet.[1] Although these were first identified as of chromophobe origin, transplantation studies suggest that they arise from A_2 acidophils.[22] The tumors of the athyroid birds may have their origin in the thyroidectomy cells, but the neoplastic cells are smaller and have less abundant cytoplasm, and specific granules are sparse and difficult to stain.

Comment

The high incidence of spontaneous pituitary tumors in the parakeet makes it difficult to evaluate the role of radiothyroidectomy in producing these tumors. In Table 3 are listed all neoplasms found at autopsy in the treated and control birds. The incidence of none of these tumors, except that of the pituitary, is thought to be affected by thyroidectomy, and yet their number is also increased in the I^{131}-treated birds. This is probably the result of chance and, coupled with the small number of pituitary tumors found, suggests that the latter are not related to the athyroid state induced by I^{131}.

Goiters, sometimes of massive size, occasionally are found in parakeets, but rarely is there an associated pituitary tumor.[23] As corollary to the study of the effect of radiothyroidectomy on the parakeet pituitary, 18 normal birds were placed on 0.1% thiouracil in drinking water supplied ad libitum. Of 13 that survived over a year, none developed a pituitary tumor. Thyroid and pituitary weights were recorded in six of these birds. The average thyroid weight of five was 26.5 mg. (normal, 6.0 mg.); the sixth bird had bilateral goiters with a combined weight of 193 mg. The epithelium in all thyroids of thiouracil-treated birds was markedly hyperplastic. The weight of the pituitaries in these parakeets averaged 1.17 mg., and the range was 1.05 to 1.3 mg.; in the controls the average pituitary weight was 0.77 mg.

TABLE 3.—*Tumor Incidence in 72 Treated and 128 Control Birds*

	Birds	
	Treated (I^{131})	Control
Pituitary tumor	3	0
Adenocarcinoma, kidney	3	3
Fibrosarcoma, kidney	1	1
Fibrosarcoma, spleen	2	1
Fibrosarcoma, liver	0	1
Fibrosarcoma, marrow	0	1
Cystadenoma, ovary	1	0
Squamous carcinoma, skin	0	1

From this it may be concluded that in the parakeet destruction of the thyroid by I^{131} or interference with the production of thyroxin by the administration of thiouracil leads to changes in pituitary cytology and to hyperplasia of the gland. However, no pituitary tumors are induced, and the high incidence of spontaneous pituitary tumors in this bird cannot be accounted for on the basis of any pituitary-thyroid imbalance.

Summary

Parakeets were given an intramuscular injection of 300μc. of I^{131}. Within five days the thyroids were hemorrhagic and necrotic; after three weeks they were wholly replaced by fibrous tissue. Changes in feather structure and color appeared two months later. Body temperature and metabolic rate were reduced.

After 70-400 days the mean pituitary weight of treated birds was 1.06 mg.; at 401-800 days it was 1.29 mg., compared to 0.92 mg. and 0.72 mg., respectively, for the controls. The pituitary of athyroid birds contained many large pale cells, resembling the thyroidectomy cells of other birds and mammals.

In 3 of 72 I^{131}-treated birds pituitary tumors were found at autopsy; however, the frequent occurrence of spontaneous pituitary tumors in parakeets suggest that radiothyroidectomy was not a causal factor.

Birds on 0.1% thiouracil developed hyperplastic thyroids; the pituitary changes

were similar to those following injection of I^{131}.

It is concluded that despite the high incidence of spontaneous pituitary tumors in the parakeet, neoplasia of this gland is not induced by an athyroid or hypothyroid state.

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Antigen-Induced Changes in Lymph Node Metallophilia

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It has been known for some time that the cytoplasm of reticuloendothelial cells, the microglia of the brain, and the blood monocytes may be stained selectively by ammoniacal-silver techniques. Marshall reviewed the subject in a recent monograph and suggested the term metallophil to designate such cells.[1] It appeared that the technique might be an aid in studies of lymph node reactivity.

The method as outlined by Marshall utilized relatively thick tissue sections which were handled without mounting on a slide. In addition, he indicated that frozen sections were preferable to paraffin sections. In view of the lack of fine detail when thick sections are studied and the nuisance of handling individual unmounted specimens, we decided to test the procedure as applied to routine slide-mounted sections cut at 5μ. It was found that clear-cut reproducible stains, free of artifactual silver deposits, could be obtained if one repeatedly washed the slides after exposure to the ammoniacal silver and the reducing formalin solution.

With this procedure we readily confirmed the metallophilic staining of the sinusoidal cells of lymph nodes, as well as scattered pulp cells which had the appearance of reticular cells. Variations in the number and form of such metallophils were clearly demonstrated. The details of such changes will be the subject of another report. At this time we should like to call attention to the unexpected finding that in some instances the lymph nodes were reproducibly stained in an entirely different fashion. In such cases the nuclear structure of the lymphoid cells stained intensely, while the metallophil cells had normal, altered, or absent staining. Such changes were found to be associated with infectious processes or antigenic stimulation. While the mechanism of these changes in ammoniacal silver staining is not clear, the distinctive nature of the findings merits a report at this time.

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From the Department of Pathology, New York Medical College, Flower and Fifth Avenue Hospitals; Professor of Experimental Pathology (Dr. Black) and Professor and Director of the Department of Pathology and Clinical Pathology (Dr. Speer).

Material and Methods

Lymph nodes from humans and mice were fixed in 10% neutral formalin, and routine 5μ paraffin sections were prepared and mounted on slides in the conventional manner. After removal of the paraffin and rehydration of the sections they were treated as follows:

1. Place in ammoniacal-silver solution, with gentle agitation for five seconds.
2. Wash in five changes of distilled water.
3. Place in 3% neutral formalin for two minutes. Agitate gently.
4. Wash in three changes of distilled water.
5. Dehydrate with alcohol.
6. Clear in xylene.
7. Mount in balsam.

The ammoniacal-silver solution was prepared as follows: Add 10% aqueous solution of silver nitrate to 3 to 4 ml. of concentrated ammonium hydroxide until the first distinct permanent turbidity is obtained. Constant stirring should be maintained during the addition of the silver nitrate solution. Approximately 38 ml. of silver nitrate solution is required if 4 ml. of ammonium hydroxide is used. The ammoniacal-silver solution was prepared each day. All staining was done in conventional Coplin jars.

Results

Control Mice.—The axillary, brachial, and inguinal lymph nodes were removed from apparently normal mature mice which had been killed by stretching the cervical cord. This series included 13 CFW females, 6 CFW males, and 10 C3H females.

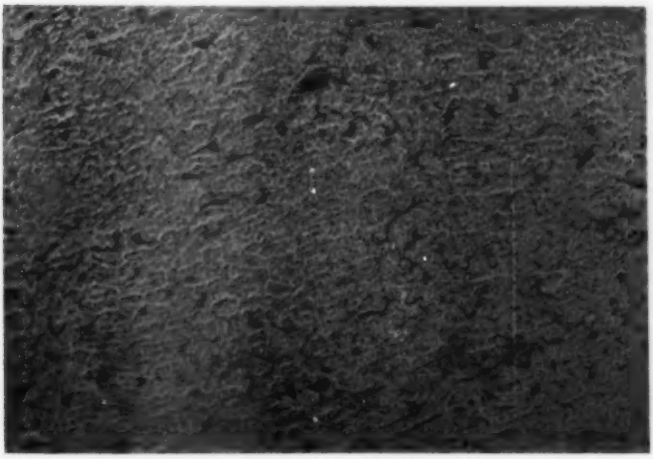

Fig. 1.—Axillary lymph node, CFW control mouse. Note that staining is confined to the cytoplasmic processes of the reticular cells (metallophil type pattern); ammoniacal-silver technique.

In the majority of the mice in each group the silver staining was similar in the axillary, brachial, and inguinal lymph nodes. The sections were characterized by metallophilic cells having finely branched cytoplasmic processes in the pulp region. In addition, the sinusoidal phagocytes and littoral cells were usually stained. No distinct staining was found in the lymphoid cells. These findings corresponded to the expected type of staining as described by Marshall and others. We shall refer to such staining as the metallophil type (Fig. 1).

In a smaller number of mice, one or all of the three node groups revealed an entirely different type of staining. Here the staining was confined to the nuclei of the lymphoid cells and so the section appeared as if it had been stained with iron hematoxylin. In such instances the reticular-cell staining was usually absent. We shall refer to this type of staining as the nuclear type (Figs. 2 and 3).

Of the total control group of 29 mice, 22 had the metallophilic type of staining in all the nodes (76%), while 2 had nuclear staining in all the nodes (7%). In the remaining five mice there was a difference in the type of staining between the axillary-brachial and the inguinal lymph nodes.

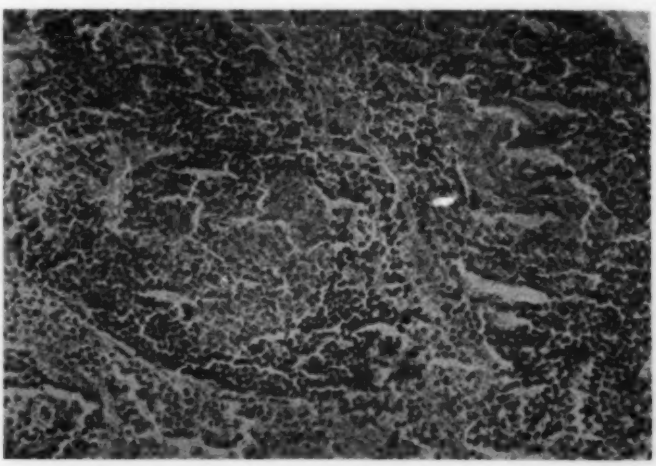

Fig. 2.—Axillary lymph node, CFW mouse six days after injection of antigen. Note the nuclear staining, in contrast to Figure 1; ammoniacal-silver technique.

Fig. 3.—Inguinal lymph node from same mouse as in Figure 2. Note that both the axillary and the inguinal nodes had nuclear type of stain; ammoniacal-silver technique.

Hematoxylin and eosin staining revealed that lymph nodes with the nuclear type of stain usually had prominent secondary follicles and plasma-cell aggregates. Such microscopic features are commonly considered to be associated with antigenic stimulation. We therefore tested the effect of antigenic stimulation on lymph node metallophilia.

Antigen-Stimulated Mice.—A series of 59 control CFW mice were given injections of foreign protein fractions, and the lymph nodes were studied at varying times after single and multiple injections. The antigenic materials included 1% solutions of human plasma protein Fractions III, IV, and IV-4 in isotonic saline as well as a solution of Fraction III which had become contaminated with a fungus growth (FCP). Injections were made intraperitoneally in some animals and subcutaneously in others.

Despite the diversity of the antigenic material and the routes of administration there was a distinct similarity in the staining reactions of the lymph nodes from the mice given injections.

Single Injection.—The staining reactions as observed after a single injection of antigen may be generalized as follows.

Within three hours in some instances and almost always within 24 hours after injection of the antigen there was a nuclear type of stain in the lymphoid cells of the cortex of the lymph nodes. At the same time there was some loss of the metallophil type of staining. The change in the silver staining preceded any microscopic change demonstrable with hematoxylin and eosin staining. Within three to four days after the antigen injection the nuclear staining was very prominent. Mitotic figures were sharply stained, as were the cells of the developing secondary follicle. After 7 to 10 days the nuclear stain was usually very prominent (Figs. 2 and 3).

Repeated Antigen Injections.—The lymph nodes of CFW mice killed as long as three weeks after receiving three weekly injections of the FCP revealed a strong nuclear stain (Figs. 4 and 5). Duplicate sections stained with hematoxylin and eosin revealed marked plasma-cell aggregates in the medullary region. The nuclei of these cells were strongly stained with ammoniacal silver.

Approximately one month after the last of three such injections of antigen the nuclear stain was much less prominent or almost gone, while the metallophilic type of staining had returned. Such metallophilic staining was usually more prominent and of a somewhat different type than in the control lymph nodes.

The following protocols illustrate the effect of antigenic stimulation on the ammoniacal-silver staining of lymph nodes.

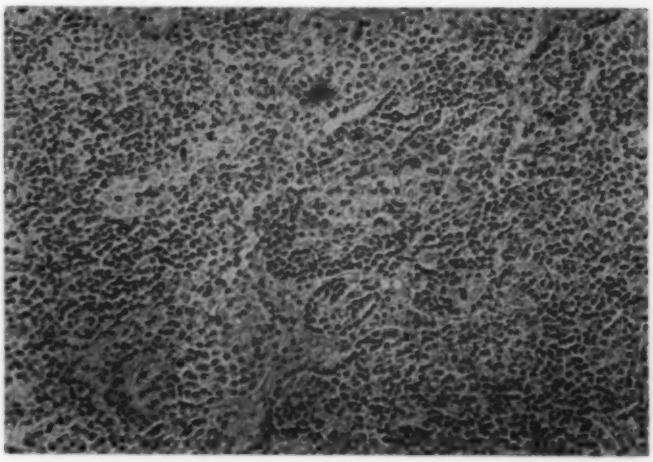

Fig. 4.—Axillary lymph node, CFW mouse given injection of antigen 35, 28, and 21 days before killing. Nuclear type staining of lymphocytes and plasma cells; ammoniacal-silver technique.

Three female CFW mice were given injections intraperitoneally of 0.1 ml. of the FCP solution. The injections were given 26, 33, and 40 days before the animals were killed. Three hours before killing one of the mice was given 0.1 ml. of isotonic saline intraperitoneally; one of the mice was given 0.1 ml. of the antigen solution intraperitoneally, while the third mouse received no additional injection. The lymph nodes of the mouse which had received the additional antigen injection had a nuclear type of stain, while the lymph nodes of the other two mice had a metallophilic type of stain.

In another study, two female CFW mice were given 0.1 ml. of the FCP solution 26, 33, and 40 days before killing. Three days before killing an additional 0.02 ml. of the antigenic solution was injected subcutaneously into the midtail region. In both of these mice the inguinal lymph nodes revealed a nuclear type of stain, while the axillary nodes had a metallophilic type of stain.

It is pertinent to note that the spleen also showed a change from the metallophilic to the nuclear type of stain after antigenic stimulation (Figs. 6 and 7).

Human Lymph Nodes.—Silver staining was performed on a series of human lymph nodes removed surgically and at autopsy from adult patients without cancer. The

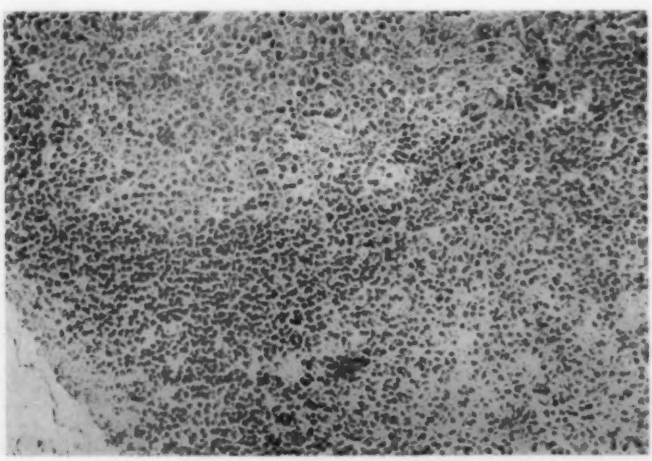

Fig. 5.—Inguinal lymph node from same mouse as in Figure 4; ammoniacal-silver technique.

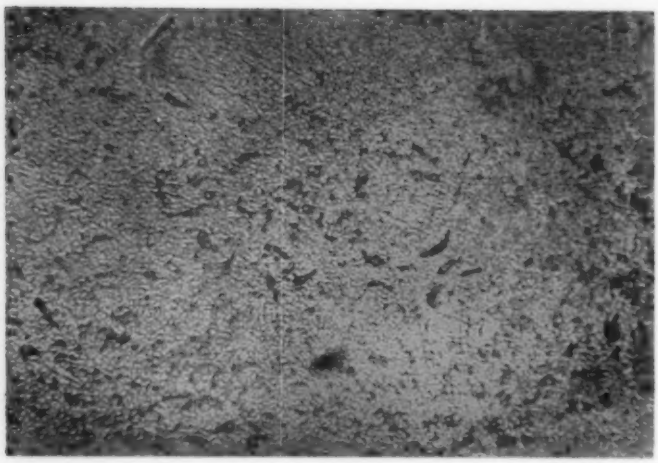

Fig. 6.—Spleen, CFW control mouse. Staining similar to that of control lymph node (metallophil type); ammoniacal-silver technique.

surgical specimens included non-neoplastic lymph nodes removed for diagnostic purposes as well as nodes removed in the course of resections of other tissues (14 cases). At autopsy the axillary lymph nodes were obtained from a series of 37 consecutive noncancerous adults. In seven of these cases the inguinals were also obtained. The number of axillary lymph nodes examined in each case ranged from 6 to 20. In the majority of cases all of the nodes from the same case had the same type of silver stain. Furthermore, in those cases where the inguinal lymph nodes were available their staining reactions were similar to that of the axillary nodes. A well-defined nuclear type of staining was found in nine of the autopsy cases and in eight of the surgically removed nodes.

When the autopsy cases were grouped according to the type of silver staining of the axillary lymph nodes it was found that the nuclear-stained group included almost all of the cases with acute infectious diseases, namely, pneumonia, four cases; lung abscess, 1 case; pyelitis, one case; peritonitis, one case (Fig. 8). In addition, this group included one case each of myocardial infarction and uremia secondary to sulfadiazine crystals. In contrast, the cause of

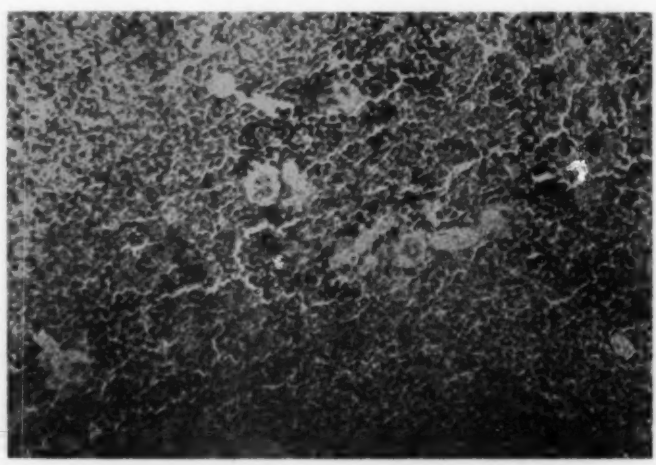

Fig. 7.—Spleen from CFW mouse six days after injection of antigen. Intensely stained nuclei are plasma cell nuclei; ammoniacal-silver technique.

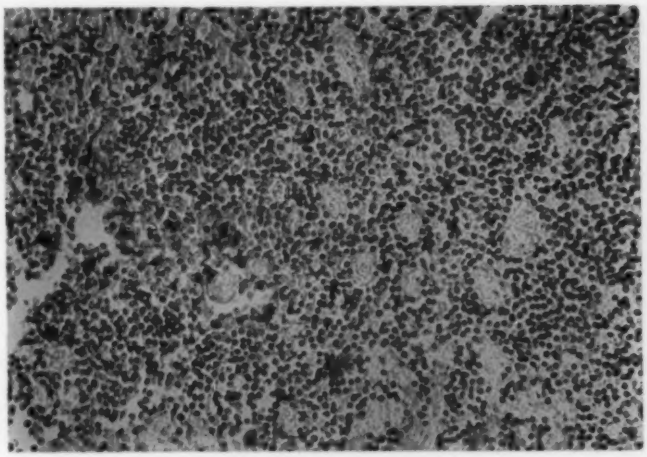

Fig. 8.—Axillary lymph node obtained at autopsy from patient dying of pyelitis. Note strong nuclear type stain; ammoniacal-silver technique.

death in those cases with metallophilic staining of the axillary lymph nodes was usually not acute infection (Fig. 9); the deaths were classified as acute deaths (postoperative, cerebrovascular accidents, myocardial infarction), six cases; congestive heart failure or uremia, eight cases; degenerative diseases (dermatomyositis, cirrhosis, amyotrophic lateral sclerosis), five cases. In addition, there were two cases which died of bronchopneumonia.

Seven additional patients had died of pulmonary tuberculosis. Their axillary lymph nodes stained in the metallophilic fashion. However, in two of these cases there was an associated weak nuclear type of stain.

The lymph nodes obtained as surgical specimens also revealed a relationship between the type of silver staining and the underlying pathology. Eight of the nodes had a nuclear type of stain (Fig. 10). These included the regional nodes from patients with peptic ulcer, three cases; appendicitis, two cases, and cholecystitis, one case, as well as a cervical lymph node draining an area of dermatitis and a retropubic node (idiopathic enlargement). The diagnosis in the six cases having a metallophilic type of staining were superficial gastritis, esopha-

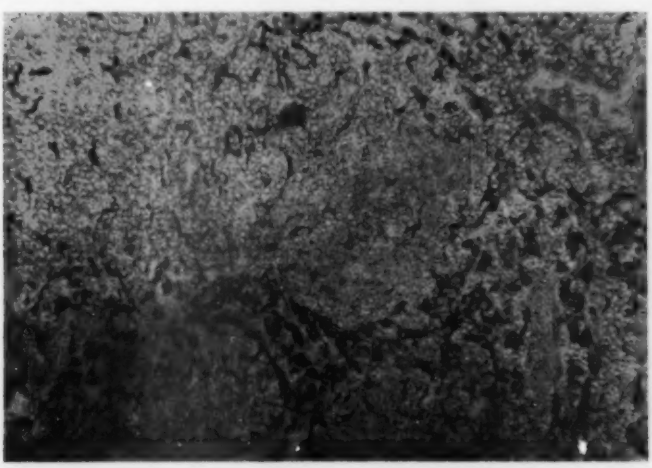

Fig. 9.—Axillary lymph node obtained at autopsy from patient who died acutely after a repair of hernia. Note strong metallophilic type of stain; ammoniacal-silver technique.

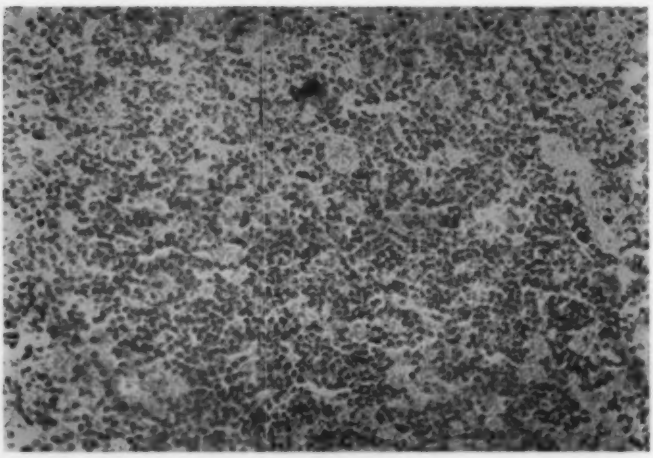

Fig. 10. — Perigastric node from patient with penetrating peptic ulcer; ammoniacal-silver technique.

geal stricture (old), and idiopathic enlargement of supraclavicular inguinal, cervical, and a femoral node removed at the time of a vein ligation.

It should be pointed out that in the human superficial lymph nodes the metallophilic cells were much more prominent than in the visceral nodes. Even in the presence of a nuclear type staining of the peripheral nodes, sinusoidal metallophilia persisted.

Comment

Although the present study is introductory, it does indicate that the ammoniacal-silver technique as described here is capable of demonstrating certain features of lymph node reactivity in a simple and reproducible fashion. In both the mouse and the human lymph nodes the finding of a nuclear type stain appeared to be related to antigenic stimulation.

We are aware that silver stains are commonly considered to be capricious. Nevertheless, in the present study the technique proved capable of yielding reproducible and predictable results on replicate testing. The underlying mechanism and significance of the observed changes in staining requires further study. However, it appears that the findings do bear a real relationship to biological events in lymph node reactivity. These changes precede the appearance of microscopic features which are conventionally related to antibody formation, i. e., follicle formation and plasma-cell aggregations. This technique may prove to be of value in clarifying the still perplexing role of the lymphocyte in immunological reactions. Detailed studies are in progress to define the sequential relationship between nuclear type staining and antibody production.

The appearance of a nuclear staining of the lymphoid cells cannot be simply a reflection of proliferation of lymphoid cells, since no nuclear staining was found in the neoplastic lymphoid cells of lymphosarcoma and Hodgkin's disease.

The main purpose of this report was to call attention to the nuclear type of staining which seems to be related to antigenic stimulation of the lymph node. However, it should be mentioned that there are also variations in the appearance of the metallophilic cells of the pulp and sinusoids in different disease processes. The details of such variations will be presented in subsequent reports.

Department of Pathology, New York Medical College, Flower and Fifth Avenue Hospitals (9).

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Atypical Epithelial Changes in the Seminal Vesicle

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It is generally accepted that very little is known about the cytology of the seminal vesicle, particularly as related to the problem of recognizing cells coming from this organ on the smears made for the detection of prostatic carcinoma. However, it is repeatedly mentioned that some of the confusions and pitfalls in prostatic cytology are derived from the presence of atypical cells thought to be from the seminal vesicles. Ackerman[1] states that "cells exfoliated from the seminal vesicles may be extremely bizarre." In his new cytology monograph, Papanicolaou[2] makes the following comment: "The exfoliative cytology of the seminal vesicle as well as that of the epididymus and the seminal duct and the extent of exfoliation occurring in these organs are practically unknown. It is therefore very difficult to identify cells of such a derivation in either the urine or the ejaculate." Peters and Frank[3] have found in direct smears from the epithelium of the seminal vesicle some bizarre and markedly atypical cells which they think are related to certain atypical cells seen in prostatic secretion smears. No description of these cells is given.

While studying the occurrence of hypertrophic nuclei in the endometrial epithelium in association with the presence of chorionic tissue, we became interested in the study of nuclear hypertrophy of benign character in various organs. Thus we noted that in some seminal vesicles large hyperchromatic nuclei occasionally were present in the epithelium.[4] At the pathology laboratories of the Memorial Center for Cancer and Allied Diseases, in New York, there has been awareness of this change.

The lack of any study defining this alteration and the above-indicated need of better information on the cytology of the seminal vesicle prompted a correlated study of the histology and cytology of this organ.

Material and Method

We have studied 152 pairs of seminal vesicles from autopsies or from surgically resected specimens. The tissues were fixed in 10% formalin, and three or more blocks were made from each vesicle. In 66 cases smears were made before fixation by gently scraping the mucosa. These smears were processed and stained by the Papanicolaou method. The age of the patients ranged from 1 week to 90 years, as follows: 1 week to 15 years, 20 cases; 16 to 30 years, 14 cases; 31 to 45 years, 32 cases, and 46 to 60 years or over, 86 cases.

This study has been carried on at the pathology laboratories of the Memorial Center for Cancer and Allied Diseases, New York; at the D. A. Carrion Hospital of Bellavista, and at the Children's Hospital of Lima, Peru.

Results

In current textbooks of histology the epithelium of the seminal vesicles is described as pseudostratified, consisting of a layer of round basal cells and of a layer of larger superficial or low columnar cells. The basal cells have a pair of centrioles above the nucleus, while in the superficial cells the centrioles are located at the surface. The cells contain numerous granules of lipochromic pigment after puberty. Although great individual variations are noted, no mention is made of the presence of isolated hypertrophic nuclei.[5-7]

The present study has demonstrated the occurrence of an epithelial change which departs from the descriptions of the normal seminal vesicle histology. The change is

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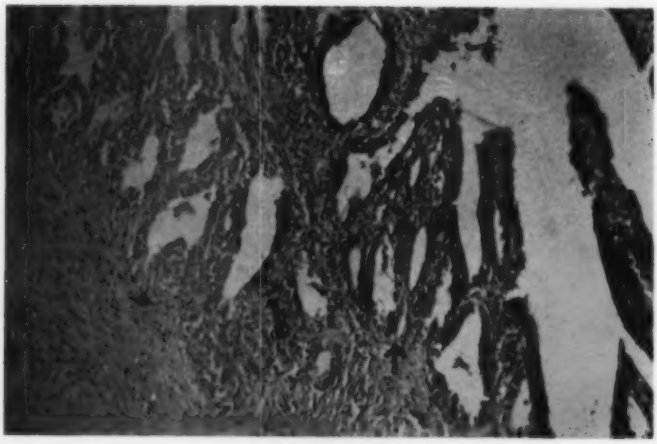


Fig. 1.—Low magnification, showing an area in which there are at least two hypertrophic nuclei (arrows). The contrast of these large nuclei with their neighbors is striking. Observe that they are normally located. Hematoxylin and eosin; reduced 35% from mag. × 140.

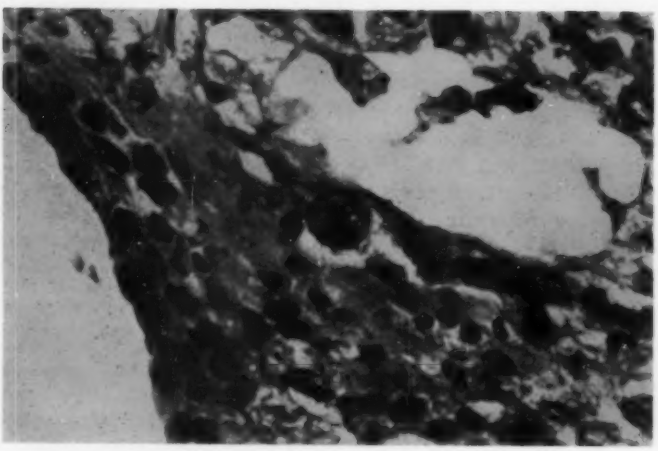


Fig. 2.—A high magnification from Figure 1, showing the nucleus at the lower center. The hypertrophic spherical nucleus displays a well-defined border, rich chromatin, and a size at least six times that of the nuclei in its vicinity. Hematoxylin and eosin; reduced 35% from mag. × 500.

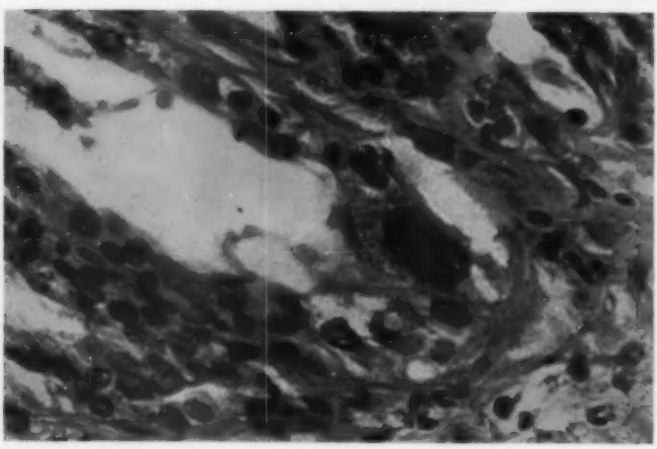


Fig. 3.—Detail of the other enlarged nucleus from Figure 1. This one is even larger and has an elongated shape and chromatin which is becoming compact and homogeneous. Note the presence of lipochromic granules on the free cytoplasmic border. Hematoxylin and eosin; reduced 35% from mag. × 500.

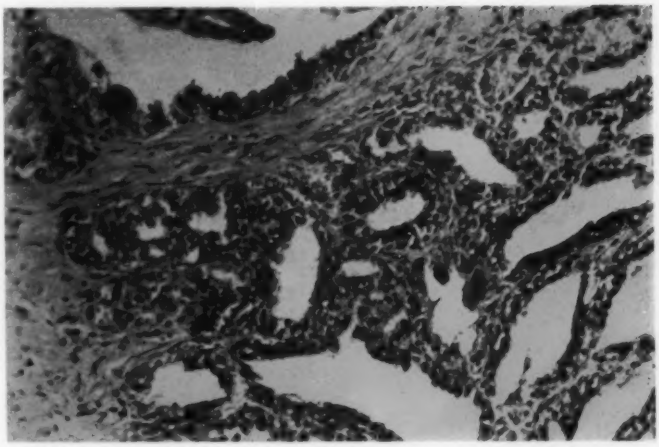


Fig. 4.—Scattered throughout the field, several large pycnotic nuclei, with bizarre shape, can be seen. A moderate degree of epithelial hyperplasia is also present. Hematoxylin and eosin; reduced 35% from mag. × 500.

characterized by focal areas in which there is nuclear hypertrophy, sometimes remarkable, of isolated epithelial cells. These areas frequently display an accentuation of the tubular arrangement, giving the impression of some degree of epithelial hyperplasia. When this change is seen in well-preserved material one can study the different aspects adopted by the enlarging nuclei and the sequence of events in their formation and evolution. The first step seems to be a process of definite increase in the chromatin content, which leads to hyperchromasia and nuclear enlargement. At this stage a well-defined nuclear membrane and a rich densely granular chromatin is noted. These cells have the general appearance of the polyploid nuclei seen in other tissues, and their presence is against interpreting them as the result of merely degenerative swelling (Fig. 2). In the next step the nucleus becomes larger and the chromatin begins to show a more compact appearance (Figs. 1, 3, and 4). Finally, a huge completely homogeneous nucleus with ill-defined borders and bizarre shape is formed (Fig. 5). As a rule the protoplasm shows lipochromic granules. Occasionally cytoplasmic vacuolation can be noted. Throughout this study no mitoses have been observed in the hypertrophic nuclei. The impression gained is that the initial increase in size is due to

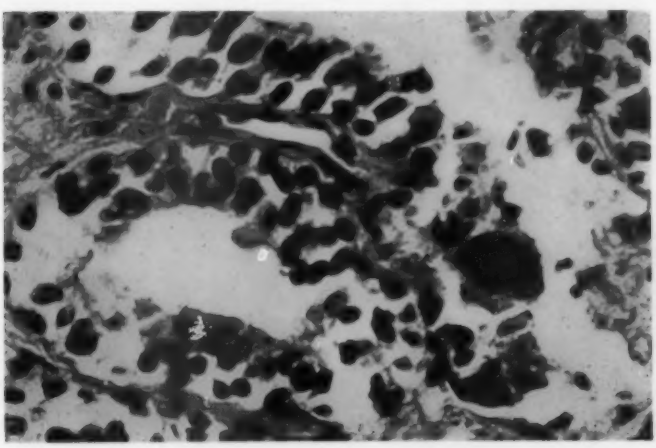


Fig. 5.—A gigantic nucleus with compact homogeneous chromatin and scanty cytoplasm. Hematoxylin and eosin; reduced 35% from mag. × 500.

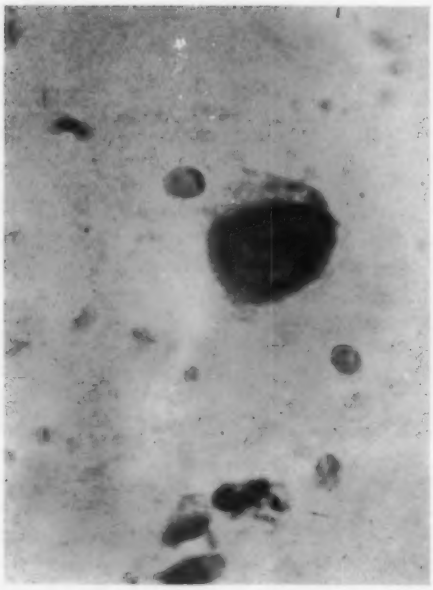


Fig. 6.—A large cell with scanty cytoplasm and huge dense nucleus. Compare the size of this cell with the group of normal cells at the lower part of the field. Papanicolaou stain.

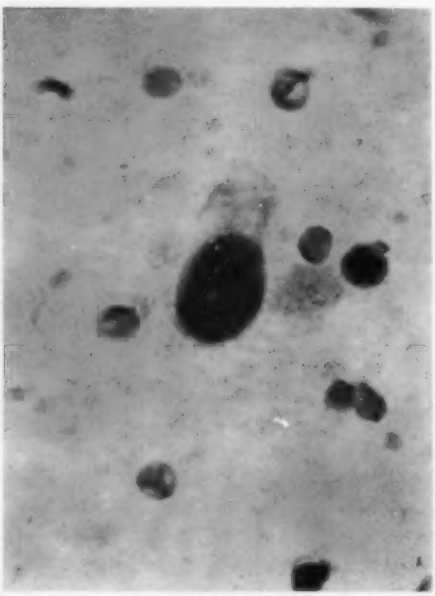


Fig. 7.—Another atypical cell, several times larger than the single normal cell present at the bottom. Papanicolaou stain.

the formation of polyploid nuclei. Later swelling and degenerative changes do occur which contribute to a greater enlargement and to the modification of the nuclear shape. A compact eosinophilic mass has been noted in some of the hypertrophic nuclei. They have the appearance of inclusion bodies, but probably represent abnormal enlarged nucleoli.

Though inflammation was seen in some sections, this did not appear to be the cause of the nuclear changes observed.

On the smears the atypical cells show marked disproportion in the cytoplasm-

Fig. 8.—One atypical cell in which granules of lipochromic pigment can be noted. Observe that in this cell as well as in the two previously seen the chromatin is of swollen homogeneous appearance, corresponding to the last step of evolution referred to in the histological description. Papanicolaou stain.

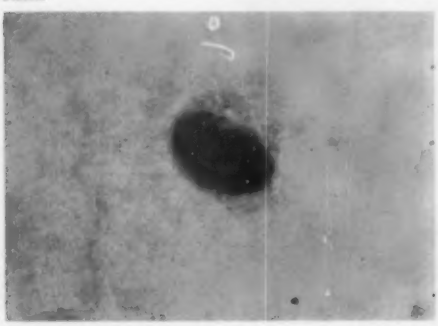
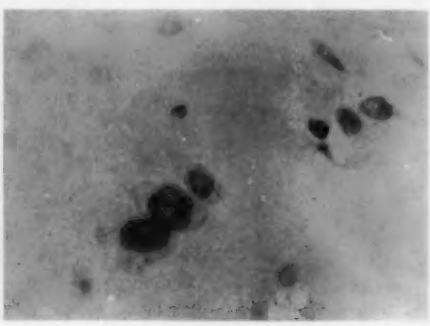


Fig. 9.—Three moderately to markedly enlarged cells and three normal cells are illustrated. The difference in size and the hyperchromasia of the atypical cells are demonstrated. Papanicolaou stain.



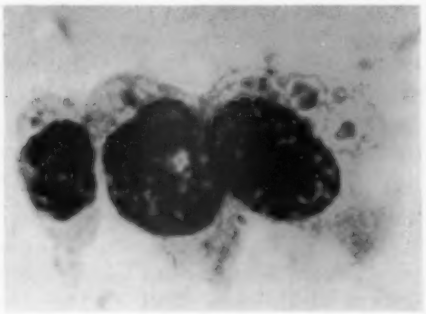


Fig. 10.—A high magnification, showing nuclear detail. Observe dense granularity of the chromatin, well-defined nuclear border, and neoplastic appearance. Lipochromic granules can be noted on the cytoplasm. Papanicolaou stain.

nuclear ratio, conspicuous lipochrome granules, and enlargement up to 20 times that of the normal cells. All the stages from slightly enlarged to gigantic cells with compact hyperchromatic nuclei can be seen (Figs. 6-10).

Well-preserved cells in the initial phase of enlargement are the ones more likely to be confused with carcinoma cells (Figs. 9 and 10). The fully developed picture of this alteration, as in the cases illustrated, is seen mostly after the fourth or fifth decade. From the 86 cases over 45 years, 11 showed marked change and 30 epithelial abnormalities of minor degree. Rarely, slight changes were noted in the third decade.

We have been unable to find a correlation of this change with the disease processes in each case or with any of the following variables investigated: vascular sclerosis in the walls of the seminal vesicle, lipochrome pigment, hyperplasia of the prostatic glands, and normality or atrophy of the testicles.

Comment

It has been our purpose at this time to characterize the alteration referred to in this paper, but no attempt will be made to discuss its pathogenesis. As a matter of fact, the data available give us little information in this regard.

Knowledge of this lesion is of importance for both histologists and cytologists. On surgical specimens one can wonder whether the hyperplastic epithelium with the gigantic nuclei is not a carcinoma. We know of cases in which this possibility has been considered. Although the cytological study of prostatic secretion has not become as popular as other cytologic investigations, there are many authors interested in this field.[8-10] Appreciation of the cells described in this paper is essential for those engaged in such studies.

Recently our attention was called to the case of a patient with hemorrhagic semen in which a diagnosis of malignant cells in the seminal secretion had been made in another laboratory. Actually the cells interpreted as carcinoma were the benign atypical cells now reported.

Summary

The morphological characteristics of an atypical epithelial change occurring in the seminal vesicle is described. Focal areas in which there is marked nuclear hypertrophy and some degree of hyperplasia in the epithelium are the main features of the alteration. The exfoliated enlarged cells can be confused with carcinoma cells. The lesion occurs mainly after the fourth decade. Inflammation and vascular sclerosis in the seminal vesicle, hyperplasia of the prostatic glands, normality or atrophy of the testicles, or the disease processes in each case were found apparently not related to the presence of the change.

Alcanfores No. 611, Miraflores.

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A Combined Periodic Acid-Schiff Trichrome Stain

SYDNEY S. LAZARUS, M.D., M.Sc. (Med.), Brooklyn

Over the past few years in various publications a periodic acid-Schiff (P. A. S.) trichrome stain has been mentioned as being used for the localization of the specific cellular sites of glycogen deposition in the pancreas.[1-6] This method is essentially a modified Masson trichrome stain,[7-12] superimposed on the P. A. S. technique,[13] and was evolved because of the tinctorial nonspecificity of Pearse's trichrome P. A. S.,[14] which had originally been used. It provides a method of high specificity which may with advantage be substituted for the ordinary Masson trichrome strain and is useful for studies of the anterior pituitary and kidney as well as for the pancreas. The method of processing the tissues as well as the staining techniques are given in detail, since both are important for good results.

Method

A. Fix tissues for 16 to 20 hours in Zenker's solution to which 20% of neutral formol (40%) is added just prior to use. The Zenker's solution consists of 5% mercury bichloride and 2.5% potassium bichromate in distilled water. The 40% formaldehyde (reagent quality) is neutralized with sodium carbonate to pH 7 and allowed to stand over marble chips.[8-10]

B. Wash in running water for at least 24 hours.

C. Process in the Autotechnicon as follows:

Absolute Alcohol	Toluene	Paraffin
Hr.	Hr.	Hr.
1. 1½	1. 1½	1-2
2. 1½	2. 1½	2-2
3. 1½	3. 1½	
4. 1½	4. 1½	
5. 1½	5. 1½	

The tissues are then placed in a third paraffin bath in the oven for 4 to 12 hours or in a vacuum oven for 2 hours. The paraffin used consists of "Tissue Mat"—melting point 56 C—which is filtered prior to use.

D. Cut sections at 2.0μ to 5.0μ as desired and float on a water bath containing dissolved gelatin. The water bath is prepared by filtering into it 1.0 to 1.5 gm. of gelatin previously dissolved by heating in a small amount of distilled water.[9,15] The sections are mounted on acid-cleaned slides which are drained of excess fluid and then dried, first on a hot plate at 45 C for 5 to 15 minutes and then in an oven containing formol vapor at 45 C for 4 to 16 hours.

E. Staining Technique.

1. Hydrate.
2. Treat with 1% iodine alcohol for 10 minutes.
3. Rinse with distilled water.
4. Decolorize with 5% sodium thiosulfate solution for one minute.
5. Wash in running water for 5 to 15 minutes.
6. Oxidize for 20 minutes in 0.6% aqueous periodic acid solution.
7. Place in Schiff reagent[16] for 20 minutes.
8. Rinse for one-half minute each in three changes of 0.5% aqueous sodium metabisulfite solution.
9. Wash in running water for 5 to 15 minutes.
10. Rinse in distilled water.
11. Stain for 10 minutes in Weigert's acid iron chloride hematoxylin which is at least 24 hours and not more than 10 days old. The Weigert's hematoxylin consists of an equal part of a 1% solution of hematoxylin in 95% alcohol added to a mixture consisting of 4 cc. of aqueous 29% ferric chloride, 1 cc. of concentrated hydrochloric acid (sp. gr. 1.18), and 95 cc. of water. The 29% ferric chloride solution is prepared with 48 gm. of $FeCl_3$-$6H_2O$ per 100 cc.[10]
12. Rinse in 95% alcohol.
13. Differentiate in 1% hydrochloric acid in 95% alcohol.
14. Wash in running water for 10 minutes.
15. Stain for two hours in a mixture of 0.2% ponceau and 0.1% orange G in 1% acetic acid.
16. Rinse in distilled water.
17. Differentiate with 1% phosphomolybdic acid until the D cells and collagen are colorless.
18. Rinse briefly in distilled water.

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From the Isaac Albert Research Institute of the Jewish Chronic Disease Hospital, Brooklyn, and the Department of Pathology, the Albert Einstein College of Medicine, New York.

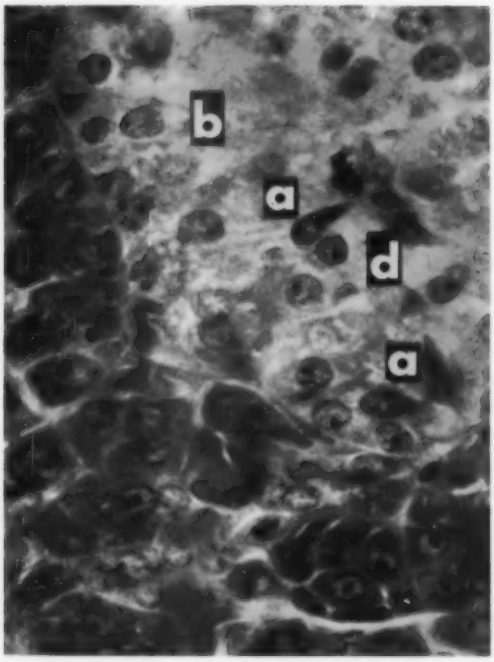

Fig. 1.—Nondiabetic human pancreas, showing islet with a-, b-, and d-cells. P. A. S.-trichrome; × 960.

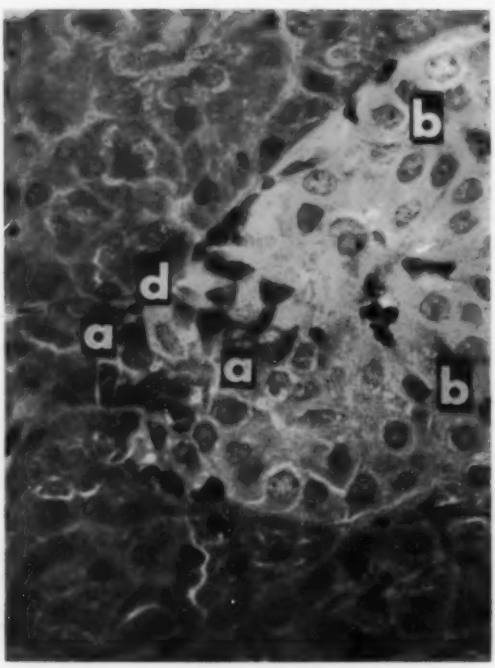

Fig. 2.—Normal rabbit pancreas, showing islet with a-, b-, and d-cells. The negative images of the Golgi apparatus' are visible as paranuclear clear spaces. P. A. S.-trichrome; × 640.

19. Stain in a 1% solution of light green in 1% acetic acid for 5 to 30 minutes.
20. Rinse well in 1% acetic acid.
21. Differentiate in 0.5% phosphomolybdic acid for one-half to five minutes.
22. Place in 1% acetic acid for 20 minutes.
23. Dehydrate rapidly by pouring absolute alcohol from a dropper bottle over each individual slide and follow by three changes of fresh absolute alcohol.
24. Clear and mount.

Results

With this method the three pancreatic islet cell types can be clearly differentiated (Figs. 1 and 2). The b-cell granules are pale yellow-orange. Their cytoplasm when visible is a pale yellowish-green color. The macular zone appears as a paranuclear homogeneous translucent green semilunar area. The Golgi apparatus is seen as clear channels. The a-cells stain a deep orange, which tends to obscure the outlines of the nucleus. Their Golgi apparatus is frequently seen as a paranuclear vacuole. The d-cells stain a homogeneous translucent green. Glycogen appears dark red and sometimes purplish, as does mucus and other glycoproteins. The ductular epithelium stains a pale grayish-green, while intraluminal secretory material is usually deep red but occasionally may be green. Nuclei are black. Collagen is green; reticular fibers and basement membranes give a positive Schiff reaction, as do the zymogen granules. Red cells stand out clear yellow.

Comment

The technique as described gives the best results only with fresh properly fixed tissue and thin sections. When the tissue is not fresh, or if improperly fixed, glycogen disappears. Furthermore, even minimal autolytic changes cause some distortion of the tissue, with changes in the staining properties. Thick sections produce opaque slides in which various colors are superimposed, making it difficult to identify structures.

For fixation of pancreatic tissue, Zenker's solution with both 10% and 20% formalin has been used.[10,17,18] However, the latter, which had been used by some as a mito-

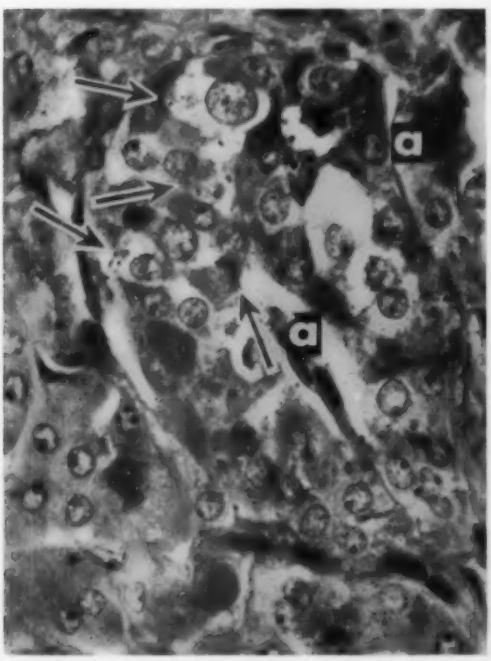

Fig. 3.—Pancreas of diabetic patient, showing intact a-cells (*a*) and glycogen (arrows) in vacuolated b-cells. P. A. S.-trichrome; × 640.

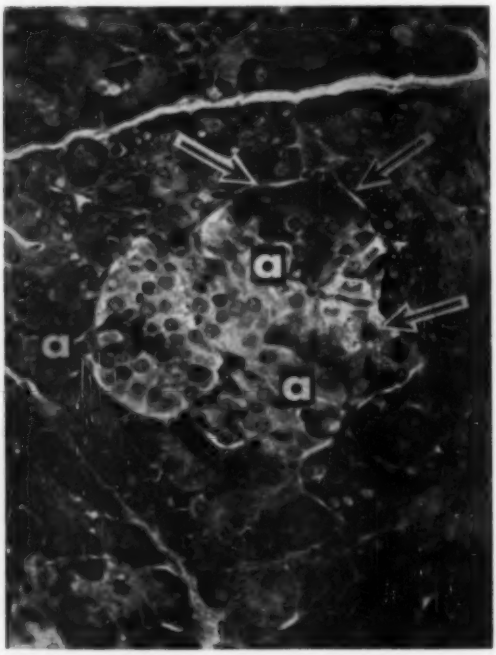

Fig. 4.—Pancreas of diabetic rabbit treated with 5 mg. of cortisone per kilogram daily for 10 days, showing heavy glycogen deposits (arrows). The a-cells (*a*) stand out clearly. P. A. S.-trichrome; × 250.

chondrial fixative, results in better cytologic detail.[9] Other fixatives, such as formalin or Bouin's solution, do not allow for the same tinctorial specificity and do not adequately preserve pancreatic glycogen.

Masson type trichrome procedures have frequently been utilized for the study of pancreatic morphology.[10,17,19-21] The combination of a trichrome stain with the periodic acid-Schiff stain provides a clarity of detail which is extremely desirable. Together with diastase digestion, it has allowed for the demonstration of specific sites of glycogen deposition within the

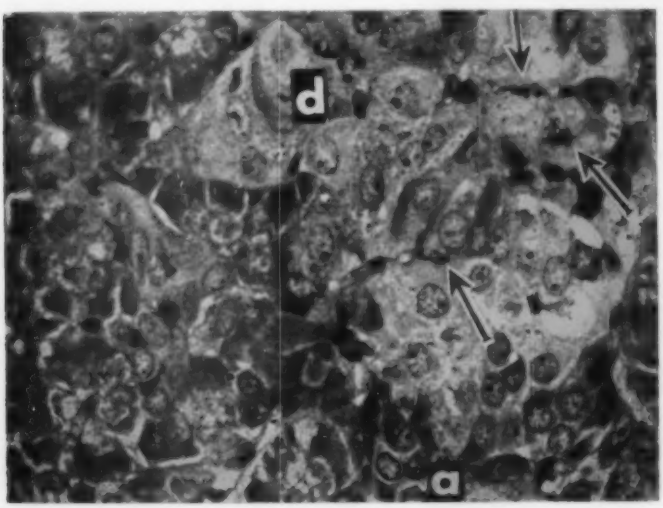

Fig. 5.—High-power view of pancreas of rabbit treated as in Figure 4 for seven days, showing islet with clearly delineated a- (*a*) and d- (*d*) cells as well as glycogen (arrows). P. A. S.-trichrome; × 640.

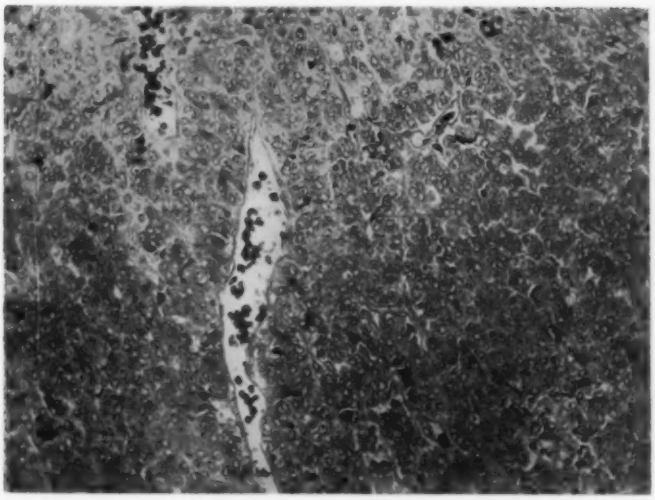

Fig. 6. — Low-power view of pancreas, difficult to identify as to whether of insular or extrainsular origin. Hematoxylin and eosin; × 190.

pancreas (Figs. 3, 4, and 5). In some instances, it has also made possible the identification of the exact cells of origin of pancreatic tumors. Pancreatic tumors of insular origin are described as showing the presence of short anastomosing cords one to three cells thick, separated by capillaries with which the cells are in intimate contact.[22] Figure 6 shows a low-power view of such a tumor which was considered after routine stains to be of islet origin. However, with the present method, it was possible to demonstrate Schiff-positive granules at the anticapillary pole of some cells as well as occasionally an acinar arrangement of cells with Schiff-positive material in the lumen (Fig. 7). This identifies the tumor as deriving from pancreatic ductular epithelium, with some differentiation to mature acinar cells.

Summary

A method is described for the processing and staining of pancreatic tissue which utilizes a combination of the periodic acid-

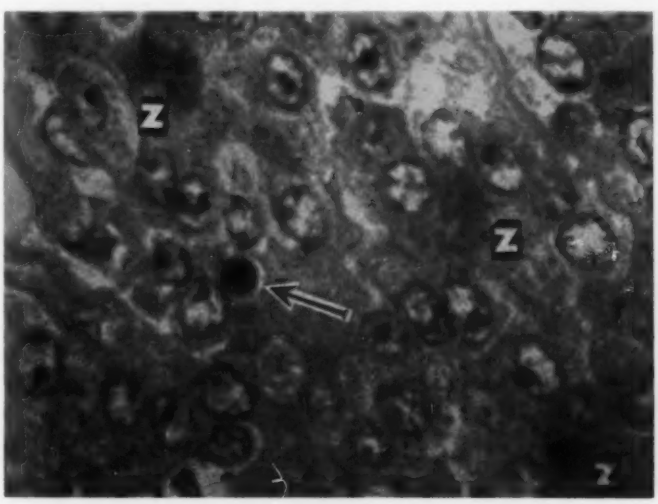

Fig. 7. — High-power view of same tumor as in Figure 5, showing P. A. S.-positive zymogen granules (*z*) arranged at anticapillary borders of cells as well as the occasional acinar arrangement of cells, with P. A. S.-positive secretory material (arrow) in the lumen. These feaures allow for the identification of this tumor as being of pancreatic ductular origin with acinar differentiation. P. A. S.-trichrome; × 1600.

Schiff (P. A. S.) technique with a Masson-type trichrome stain. With this method good cellular and cytologic detail may be obtained which allows for the identification of various cell types as well as specific sites of glycogen deposition within the pancreas. This method has also been found suitable for the study of human pancreatic tumors, the anterior pituitary gland, and the kidney.

Miss Josephine McLeod provided technical assistance. The photographs were prepared by Mr. Herbert A. Fischler.

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News and Comment

ANNOUNCEMENTS

Symposium on Cancer Research.—The Thirteenth Annual Symposium on Fundamental Cancer Research of the University of Texas M.D. Anderson Hospital and Tumor Institute, Houston, will be held on Feb. 26, 27, and 28, 1959. The topic will be "Genetics and Neoplastic Growth." For information regarding the program, inquiries should be addressed to the General Chairman: Felix L. Haas, Department of Biology, University of Texas M.D. Anderson Hospital, Houston 25, Texas.

PERSONAL

Dr. Arnold R. Rich Retires.—Dr. Arnold R. Rich retired on July 1, 1958, as Professor of Pathology, Director of the Department of Pathology, and Pathologist-in-Chief to the Johns Hopkins Hospital after 43 years of association with The Johns Hopkins University School of Medicine. He has been succeeded by Dr. Ivan L. Bennett Jr., who has been serving as Professor of Medicine.

CORRECTION

In the article "Pathogenesis of Metastasis Formation observed in Vivo in the Rabbit Ear Chamber," by Dr. Sumner Wood Jr., in the October issue of the Archives, Figure 39 (p. 562) was printed upside down.

Books

The Anatomy of Congenital Pulmonary Stenosis. By Sir Russell Brock, M.S., F.R.C.S., F.A.C.S. (Hon.). Price, $7.50. Pp. 114, with 76 illustrations. Paul B. Hoeber, Inc. (medical book department of Harper & Brothers), 49 E. 33d St., New York 16, 1957.

This small text, referred to by the author himself as a monograph, represents a well-written and authoritative study of pulmonary stenosis. The subject is logically developed from its historical and embryological origins through a classification with discussion of the various types, ending with surgical treatment and newer physiological concepts.

The role of the bulbus cordis in the formation of pulmonary stenosis is emphasized, and the comparative anatomy and embryology studies in this respect are particularly interesting. The classification of congenital pulmonary stenosis is both useful and lucid, and the descriptions of each type appear adequate. The discussion of the infundibular varieties of stenosis are particularly excellent. The physiological changes, such as reversal of shunts, both pre- and postoperative, are included.

Important correlations of findings at surgery with manometric readings and electrocardiographic tracings are described. Of importance are the author's concepts of the value of diagnosis at the operating table in spite of the use of preoperative electrocardiograms, catheterizations, or angiograms. The value of manometric pulmonary artery readings and good clinical response, important clinical points concerning the postoperative course, danger signs, and treatment of certain postoperative situations are only a few of the practical subjects discussed.

Because of the size of the text, it suffers from lack of detail. This is particularly true of the anatomic pathological findings in congenital pulmonary stenosis. For example, little histopathology is mentioned. However, of interest to the pathologist are the striking differences stressed again and again by the author between the anatomical findings of the heart at operation and in the preserved specimen.

The book is well designed, the paper and print are of fine quality, and the pictures, drawings, and charts are clear and well illustrate the points discussed. Actually, the text represents a very practical study of congenital pulmonary stenosis combining the embryology, classification, physiology, and present-day surgery concepts. It should prove most useful to the thoracic surgeon.

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